

Universidade de Lisboa
Faculdade de Ciências
Departamento de Biologia Vegetal



Study of a new pathway involved in electron bifurcation in anaerobic bacteria

Gonçalo Pizarro Madureira Salgado de Oliveira

Dissertação

MESTRADO EM MICROBIOLOGIA APLICADA

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Dissertação orientada por
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Abstract

Bioenergetics of chemotrophic bacteria is based on substrate-level phosphorylation and electron transfer phosphorylation for energy conservation. Recently, a third mechanism of energy coupling named flavin-based electron bifurcation (FBEB) was proposed for anaerobic bacteria. Sulfate-reducing organisms (SRO) are a polyphyletic group of anaerobic microorganisms, which perform dissimilatory sulfate reduction. Interestingly, studies concerning SRO identified protein homologies to enzymes engaged in FBEB in methanogens, suggesting the presence of FBEB in sulfate reducers. Here we studied: i) the physiological role of a protein complex (HdrABC-FloxABCD) possibly involved in FBEB; and ii) the function of DsrD, a protein potentially involved in sulfite reduction, the last step of the dissimilatory sulfate reduction.

Firstly, phenotypic characterization of *hdrC* and *floxA* mutants in *Desulfovibrio vulgaris* Hildenborough with ethanol as electron donor revealed no cell growth, while a complemented strain of the *floxA* mutant grew similarly to the wild-type (WT). Then, under pyruvate fermentation conditions, both mutants produced low levels of ethanol comparing to the WT and complemented strain. Gene and protein expression analysis in WT strains cultured with different electron donors/acceptors showed upregulation of FloxA and HdrA when ethanol is the electron donor. Moreover, an alcohol dehydrogenase (*adh1*) gene present upstream of the *hdr-floxA* cluster is also upregulated in the medium containing ethanol. Altogether, these results show that the HdrABC-FloxABCD complex is involved in the ethanol metabolism of *D. vulgaris*.

Secondly, the *dsrD* gene from *D. vulgaris* was cloned, overexpressed and the protein purified. Sulfite reduction activity and protein-protein interaction studies showed no direct biochemical role of DsrD in sulfite reduction. Then a *dsrD* deletion mutant was generated showing a long lag phase under sulfate reduction conditions when compared to the WT. This mutant did not grow with sulfite as electron acceptor, revealing the importance of *dsrD* in sulfite reduction, most likely at a regulatory level.

Overall, this work allowed a better understanding of energy conservation mechanisms in SRO, proposing a new mechanism for ethanol metabolism played by the FloxABCD-HdrABC complex and producing new insights into the function of DsrD in the dissimilatory sulfate reduction.

Keywords: Bioenergetics; energy conservation; flavin-based electron bifurcation; sulfate-reducing organisms; gene expression studies, phenotype characterization; protein purification.

Resumo

Os sistemas bioenergéticos de bactérias quimiotróficas baseiam-se em dois mecanismos de conservação de energia: a fosforilação ao nível do substrato (FNS) e a fosforilação oxidativa (FO). A FO é também conhecida como respiração e envolve acoplamento quimiosmótico. Recentemente, um outro mecanismo de acoplamento energético, chamado bifurcação electrónica baseada em flavinas (BEBF), foi proposto para bactérias anaeróbias quimiotróficas. Este mecanismo é caracterizado por acoplar uma reacção termodinamicamente não favorável a uma reacção favorável e foi já experimentalmente demonstrado em organismos fermentativos, arqueas metanogénicas e bactérias acetogénicas. Os complexos proteicos envolvidos na BEBF são citoplasmáticos, contêm flavinas como cofactores (FMN ou FAD) e um dos aceitadores/dadores de electrões é normalmente uma ferredoxina (Fd).

Os organismos redutores de sulfato (ORS) são um grupo polifilético de microrganismos existentes em ambientes anaeróbios, tendo um metabolismo versátil. Os sulfato-redutores têm a capacidade de realizar a redução dissimilativa do sulfato, *i.e.*, de reduzir grandes quantidades de sulfato como mecanismo de conservação de energia produzindo sulfureto, um produto tóxico do seu metabolismo. Deste modo estes organismos contribuem para o ciclo biogeoquímico do enxofre usando o sulfato como aceitador de electrões durante a degradação de matéria orgânica, produzindo sulfureto que pode ser oxidado por outros microrganismos. Pela capacidade que têm de utilizar diversos dadores e aceitadores de electrões, os ORS são usados em bio-remediação de metais tóxicos e compostos orgânicos como hidrocarbonetos; todavia estes organismos comportam igualmente um impacto negativo, nomeadamente contribuindo para a bio-corrosão de metais ferrosos e de betão e contribuindo ainda para a acidificação de reservas de petróleo. Apesar da importância ambiental destes microrganismos, os mecanismos de conservação de energia neste grupo permanecem por conhecer com clareza. Para estudos fisiológicos, genéticos e bioquímicos é normalmente utilizado o organismo modelo *Desulfovibrio vulgaris* Hildenborough, uma vez que é relativamente fácil e rápido de cultivar em laboratório, tendo sido o primeiro ORS a ter o seu genoma sequenciado. Estudos genómicos identificaram proteínas semelhantes à heterodisulfureto redutases (Hdr) em ORS, especialmente proteínas homólogas à HdrA, a subunidade proteica que contém uma flavina como co-factor e que foi proposta ser responsável pela BEBF em arqueas metanogénicas. Este estudo sugere desta forma que o mecanismo de BEBF pode estar presente em procariotas sulfato-redutores.

Assim sendo, o objectivo deste trabalho centra-se em contribuir para um maior e mais profundo conhecimento dos mecanismos de conservação de energia em sulfato-redutores, estudando o papel fisiológico do complexo citoplasmático heterodisulfureto redutase-oxidase de flavina (HdrABC-FloxABCD), que se pensa estar envolvido em BEBF, e investigando a potencial função da proteína redutase dissimilativa de sulfito (DsrD) na via da redução dissimilativa do sulfato.

Na primeira parte do trabalho experimental, estudou-se o papel fisiológico dos genes *flox-hdr* em *D. vulgaris* Hildenborough, usando duas estirpes mutantes: IPFG01, com uma cassete de resistência a canamicina inserida no gene *hdrC*, e IPFG02, uma estirpe em que o gene *floxA* foi substituído por uma cassete de resistência a canamicina por recombinação homóloga. A caracterização fenotípica das estirpes mutantes revelou que ambos os mutantes são incapazes de crescer quando o etanol é usado como dador de electrões para reduzir sulfato. Por outro lado uma estirpe mutante complementada com o gene *floxA* (IPFG03) cresceu de forma semelhante ao WT. Durante o crescimento fermentativo em piruvato, realizou-se a quantificação da concentração de etanol no meio extracelular através de um método enzimático. Este ensaio experimental revelou uma produção muito baixa de etanol por parte das estirpes mutantes IPFG01 e IPFG02, comparativamente às estirpes WT e IPFG03, indicando deste modo que em condições fermentativas de crescimento, as proteínas FloxABCD estão envolvidas na redução de NAD⁺ para produção de etanol. Foram adicionalmente realizados estudos de expressão génica ao nível do gene (por PCR quantitativo) e da proteína (por “Western blotting”) na estirpe selvagem (WT) em condições de cultura contendo diferentes dadores e aceitadores de electrões. Estes revelaram que os genes *floxA* e *hdrA* são sobre-expressos quando é usado etanol como dador de electrões, e o mesmo foi observado ao nível da expressão proteica. A montante dos genes *hdr-flox* está situado um gene que codifica para uma álcool desidrogenase (*adh1*) revelando igualmente uma sobre-expressão quando o etanol é usado como dador de electrões. Contudo a expressão do gene *adh1* é consideravelmente superior à dos genes anteriormente referidos, indicando que os genes *adh1* e *hdr-flox* não se encontram no mesmo operão. Esta diferença observada ao nível da expressão génica reflectiu-se na purificação das proteínas: enquanto que a proteína Adh1 foi facilmente purificada, o mesmo sucesso não foi obtido na purificação das proteínas Flox e Hdr devido à sua baixa expressão. Os resultados obtidos considerando esta parte do trabalho demonstram que as proteínas FloxABCD estão envolvidas no metabolismo do etanol em *D. vulgaris*. Propomos então que o complexo FloxABC-HdrABCD seja capaz de realizar BEBF acoplando a redução de Fd com NADH à redução da proteína DsrC igualmente com NADH.

Na segunda parte deste trabalho, o gene *dsrD* foi clonado, sobre-expresso em *Escherichia coli* e a proteína subsequentemente purificada por técnicas cromatográficas. O gene *dsrD* encontra-se situado imediatamente a jusante do gene *dsrAB*, fazendo ambos parte do mesmo operão. A redutase dissimilativa de sulfito (DsrAB) é, juntamente com a proteína DsrC, responsável pelo último passo da redução dissimilativa do sulfato. Tendo em conta a potencial importância da proteína DsrD na redução do sulfato, foram realizadas ensaios espectrofotométricos de actividade enzimática de redução do sulfito e testes de interação proteína-proteína (por Biacore) e proteína-ligando (por Calorimetria de Titulação Isotérmica) de modo a detectar interações entre a DsrD e DsrAB, DsrC e/ou sulfito. Apesar das diversas tentativas, não foram obtidos resultados positivos, levando-nos a crer que a proteína DsrD desempenha um papel regulatório em vez de um papel funcional directo na redução do sulfato. Um vez que a estrutura tridimensional da proteína foi já obtida e inclui um

domínio de ligação ao DNA, considera-se que a proteína DsrD desempenhe um papel regulatório *in vivo*. Durante este trabalho experimental foi construído um mutante de deleção por troca da sequência codificante para o gene *dsrD* com uma cassette de resistência a canamicina através de recombinação homóloga. Os estudos fenotípicos com esta nova estirpe revelaram uma longa fase de adaptação na curva de crescimento em meio de cultura contendo sulfato como aceitador de electrões, comparativamente com a estirpe WT. Pensamos que após a longa fase de adaptação as células adquirem espontaneamente mutações que lhes permitem adaptar-se ao meio contendo sulfato, demonstrando que o gene *dsrD* pode estar a regular os genes *dsrAB* afectando deste modo a via respiratória. Adicionalmente, a estirpe mutante não foi capaz de crescer em meio com sulfito como aceitador de electrões, o que revela que este gene é essencial para a redução do sulfito.

De uma forma geral, este trabalho contribuiu para uma melhor e mais profunda compreensão do modo como os organismos sulfato-redutores desempenham a conservação de energia, com especial interesse na nova via de conservação de energia, BEBF, que aparenta estar disseminada pelas bactérias quimiotróficas anaeróbias. Em perspectivas futuras seria ideal conseguir expressar e purificar o complexo FloxABCD-HdrABC de modo a analisar a BEBF *in vitro*. Adicionalmente, novas indicações foram obtidas no que toca a compreender qual a função ao nível fisiológico da proteína DsrD, envolvida na redução dissimilativa do sulfato. Em continuação deste projecto, seria interessante determinar a capacidade de interação da proteína DsrD com o DNA *in vitro*, analisar que genes substituem a função do gene *dsrD*, permitindo a adaptação da estirpe mutante ao meio de cultura contendo sulfato como aceitador de electrões, e ainda qual a contribuição desses mesmos genes para a via da redução dissimilativa do sulfato.

Palavras-chave: Bioenergética; conservação de energia; bifurcação electrónica baseada em flavinas; organismos redutores de sulfato; estudos de expressão génica; caracterização fenotípica; purificação de proteínas.

Index

LIST OF ABBREVIATIONS	XI
1 – INTRODUCTION	XII
1.1 – Energy conservation in anaerobic bacteria	1
1.1.1 – Bioenergetic origins	2
1.1.2 – Flavin-based electron bifurcation – a new pathway for energy coupling	3
1.1.3 – FBEB examples	4
1.2 – Sulfate-Reducing Organisms (SRO)	6
1.2.1 – Sulfur cycle	7
1.2.2 – Physiology and Biochemistry	7
1.2.3 – Taxonomy	8
1.2.4 – Environmental impact	10
1.3 – The <i>Desulfovibrio</i> genus	10
1.3.1 – Morphology, biochemistry, and genome features	11
1.3.2 – Taxonomy	11
1.3.3 – Dissimilatory sulfate reduction	11
2 – OBJECTIVES	14
3 – MATERIALS AND METHODS	15
3.1 – The <i>hdr-flox</i> gene cluster	15
3.1.1 – Strains and media	15
3.1.2 – Growth curves	15
3.1.3 – Ethanol quantification	15
3.1.4 – Adh1 purification and activity measurements	17
3.1.5 – Reverse Transcriptase quantitative PCR (RT-qPCR)	17
3.1.6 – Western blot analysis of FloxA, HdrA, and Adh1 expression	18
3.2 – Studies with <i>dsrD</i>	19
3.2.1 – <i>dsrD</i> heterologous expression	19
3.2.2 – <i>dsrD</i> deletion	20

4 – RESULTS	22
4.1 – The <i>hdr-flox</i> gene cluster	22
4.1.1 – Growth curves	22
4.1.2 – Adh1 purification and activity measurements	24
4.1.3 – Reverse Transcriptase quantitative (RT-qPCR)	25
4.1.4 – Western blot analysis of FloxA, HdrA, and Adh1 expression	25
4.2 – Studies with <i>dsrD</i>	26
4.2.1 – DsrD activity and interaction assays	26
4.2.2 – Growth curves	27
5 – DISCUSSION	28
5.1 – The <i>hdr-flox</i> gene cluster	28
5.2 – <i>dsrD</i>	32
REFERENCES	33

List of abbreviations

Ack – Acetate kinase
Adh – Alcohol dehydrogenase
ADP – Adenosine diphosphate
Al-dh – Aldehyde dehydrogenase
AMP – Adenosine monophosphate
Aor – Aldehyde:ferredoxin oxidoreductase.
Apr – Adenosine-5'-phosphosulfate reductase
APS – Adenosine-5'-phosphosulfate
ATP – Adenosine triphosphate
BCA – Bicinchoninic acid assay
Bcd/Etf – Butyryl-CoA dehydrogenase/electron transfer complex
BCIP – 5-bromo-4-chloro-3-indolyl phosphate
CoA – Coenzyme A
CoB-SH – Coenzyme B, N-7-mercaptoheptanoyl-L-threonine phosphate
CoM-SH – Coenzyme M, 2-mercaptoethanesulfonate
CoM-S-S-CoB – Heterodisulfide of CoM-SH and CoB-SH
dCTP – Deoxycytidine triphosphate
DMRB – Dissimilatory metal-reducing bacteria
Dsr – Dissimilatory sulfite reductase
 E^0 – Standard electron potential
EDTA – Ethylenediaminetetraacetic acid
EtOH – Ethanol
ETP – Electron transport phosphorylation
FAD – Flavin adenine dinucleotide
FBEB – Flavin-based electron bifurcation
Fd – Ferredoxin
Flox – Flavin oxidoreductase
FMN – Flavin mononucleotide
Hase – Hydrogenase
Hdr – Heterodisulfide reductase
Hyd – [Fe]-only hydrogenase
ITC – Isothermal Titration Calorimetry
Km – Kanamycin
LA – Luria agar
LB – Lysogeny broth
Ldh – Lactate dehydrogenase
MOY – MO basal medium with yeast extract
MQ – Menaquinone

MQH₂ – Menaquinol
 Mvh – F₄₂₀ non-reducing hydrogenase
 NAD⁺ – Nicotinamide adenine dinucleotide
 NADH – Nicotinamide adenine dinucleotide reduced form
 NBT – Nitro-blue tetrazolium chloride
 Nfn – NADH-dependent reduced ferredoxin:NADP⁺ oxidoreductase
 OD – Optical density
 PCR – Polymerase chain reaction
 P_i – Inorganic phosphate
 Por – Pyruvate:ferredoxin oxidoreductase
 PP_i – Inorganic pyrophosphate
 Pta – Phosphate acetyltransferase
 PVDF – Polyvinylidene difluoride
 Qmo – Quinone-interacting membrane-bound oxidoreductase
 Rnf – *Rhodobacter* nitrogen fixation
 RT-qPCR – Reverse Transcriptase quantitative Polymerase Chain Reaction
 SLIC – Sequence ligation independent cloning
 SLP – Substrate-level phosphorylation
 SPR – Surface Plasmon Resonance
 SRO – Sulfate-reducing organisms
 TBS – Tris-buffered saline
 TBST – Tris-buffered saline Tween 20
 Tplc₃ – Type I cytochrome c₃
 Tris – Tris(hydroxymethyl)aminomethane
 UV – Ultraviolet
 Vho – Methanophenazine-reducing [NiFe] hydrogenase
 WT – Wild-type

1 – Introduction

1.1 – Energy conservation in anaerobic bacteria

Life requires energy. One of the features that distinguish living beings from inanimate objects is their metabolism. Metabolism balances energy through a set of chemical transformations occurring in two forms: anabolism, biosynthetic reactions requiring energy input; and catabolism, molecular breakdown reactions that allow to restore the cellular energy budget. The universal molecular currency of energy adopted by living beings is proton motive force, which is consumed for cellular processes and produced by energy conservation mechanisms. Additionally, there are two environmental sources for energy conservation: in chemotrophic organisms, energy derives from the oxidation of environmental electron donors, while in phototrophic organisms energy comes from sunlight (**Figure 1.1**). Chemotrophic organisms exist virtually since the dawn of life on Earth and have evolved to very distinct metabolic systems ever since.

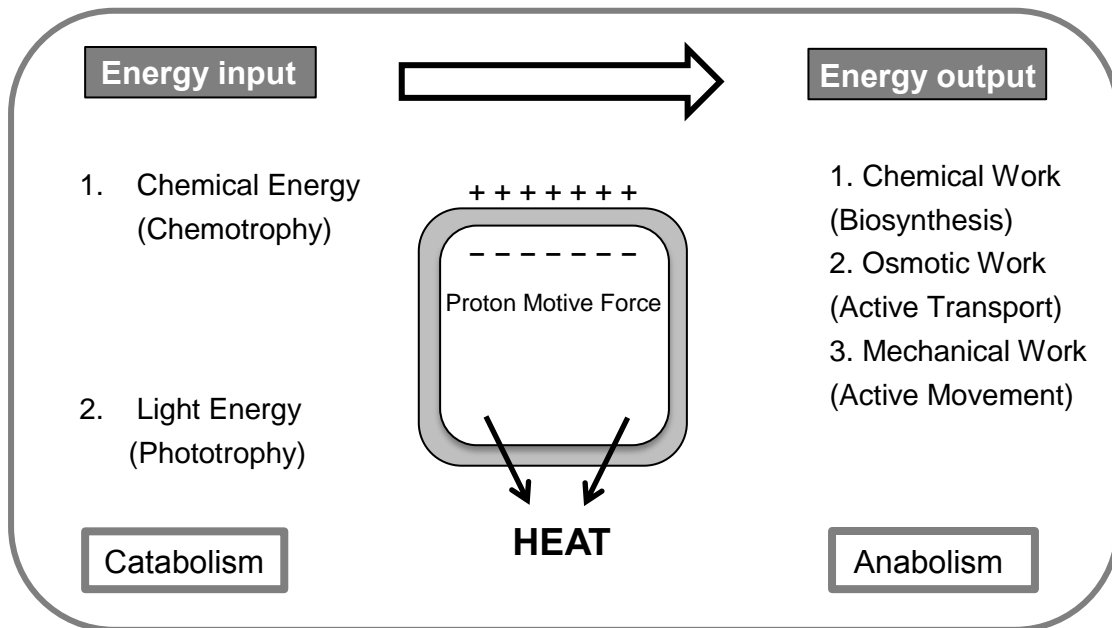


Figure 1.1 – Energy transformations via the proton motive force system. Adapted from (1).

1.1.1 – Bioenergetic origins

At the time life is estimated to have emerged on Earth (about 4.6 billion years ago) our planet had quite different geological and atmospheric characteristics from the ones observed today. The prebiotic atmosphere was mainly composed of hydrogen, ammonia, methane, carbon dioxide, and water (2). In the 1920's Aleksandr Oparin and John Haldane hypothesized that these compounds, combined with the strong UV radiation present at that time, led to the emergence of the first biomolecules in ocean waters. The combination of these molecules, along with the action of Natural Selection during millions and millions of years, formed the complex life forms we know of today.

Since the primordial atmosphere had no oxygen, the first living beings had to replicate and generate their own energy from an anaerobic environment. Energy was stored in the form of ATP, an energy-rich compound required for anabolic reactions and cell function; this biochemical signature has been conserved by nature thereafter and is still present among all living forms. In this regard, the first metabolism capable of sustaining life would use the compounds available in the primitive environment in order to generate energy for cellular biochemical reactions.

During the evolution of life, new metabolic machineries started to emerge. Despite the diversity of mechanisms, energy conservation in chemotrophs is thought to exist only via two different ways: substrate-level phosphorylation (SLP) and electron-transfer phosphorylation (ETP), also known as oxidative phosphorylation (1). In the first mechanism, energy-rich compounds form ATP by transferring a phosphoryl group to ADP in anaerobic conditions. The second mechanism (ETP) involves the formation of an electrochemical gradient (ΔpH for protons or ΔpNa for sodium ions) across the cytoplasmic membrane generated by an electron transfer chain that reduces a terminal acceptor (3). The electrochemical gradient formed drives a cationic flow through ATP synthase, which causes subunits of this enzyme to rotate, leading to conformational changes in the active site, which culminates in the formation of ATP from ADP and P_i (4). Two major types of oxidative phosphorylation, also called respiration, emerged differing in the electron acceptors used. These respiratory organisms used both SLP and ETP pathways using a diverse range of electron donors/acceptors for energy conservation (**Table 1.1**). Anaerobic respiration emerged first, using reduced compounds such as sulfur or iron as electron acceptors. After the rise of atmospheric oxygen concentration caused by the metabolism of photosynthetic organisms, aerobic respiration emerged using oxygen as terminal electron acceptor. Since oxygen has a higher redox potential (E^0) than the electron acceptors used in anaerobic respiration it permits the release of more energy per oxidized molecule.

Although it is classically considered that fermentative organisms do not perform respiration, recent studies indicate that their energy conservation mechanisms may be more complex. There are several examples of an electrochemical gradient necessary for ATP synthesis being formed in the membrane of fermentative organisms, such as by electrogenic transport in lactic acid bacteria (5), electron transfer through energy-conserving

hydrogenases in hyperthermophiles (6), and also by sodium-translocating NADH dehydrogenases in glutamate fermenting bacteria (7).

Table 1.1 – Redox potential of electron donors/acceptors involved in electron transport phosphorylation. Adapted from (1).

Redox compound	E^0 (mV)	Redox compound	E^0 (mV)
$\text{SO}_4^{2-}/\text{HSO}_3^-$	–516	Flavodoxin ox/red (E^0_2)	–115
$\text{CO}_2/\text{formate}$	–432	$\text{HSO}_3^-/\text{HS}^-$	–116
H^+/H_2	–414	Menaquinone ox/red (MK)	–74
$\text{S}_2\text{O}_3^{2-}/\text{HS}^- + \text{HSO}_3^-$	–402	APS/AMP + HSO_3^-	–60
Flavodoxin ox/red (E^0_1)	–371	Rubredoxin ox/red	–57
Ferredoxin ox/red (E^0_1)	–398	Acrylyl-CoA/propionyl CoA	–15
NAD^+/NADH	–320	Glycine/acetate $^-$ + NH_4^+	–10
Cytochrome c_3 ox/red	–290	2-Demethylvitamin K_{12} ox/red	+25
$\text{CO}_2/\text{acetate}^-$	–290	$\text{S}_4\text{O}_6^{2-}/\text{S}_2\text{O}_3^{2-}$	+24
S^0/HS^-	–270	Fumarate/succinate	+33
CO_2/CH_4	–244	Ubiquinone ox/red	+113
FAD/FADH_2	–220	$\text{S}_3\text{O}_6^{2-}/\text{S}_2\text{O}_3^{2-} + \text{HSO}_3^-$	+225
Acetaldehyde/ethanol	–197	NO_2^-/NO	+350
Pyruvate/lactate $^-$	–190	$\text{NO}_3^-/\text{NO}_2^-$	+433
FMN/FMN H_2	–190	$\text{Fe}^{3+}/\text{Fe}^{2+}$	+772
Dihydroxyacetone phosphate/glycerol-phosphate	–190	$\text{O}_2/\text{H}_2\text{O}$	+818
$\text{HSO}_3^-/\text{S}_3\text{O}_6^{2-}$	–173	$\text{NO}/\text{N}_2\text{O}$	+1175
Oxaloacetate $^{2-}/\text{malate}^{2-}$	–172	$\text{N}_2\text{O}/\text{N}_2$	+1355

1.1.2 – Flavin-based electron bifurcation – a new pathway for energy coupling

In recent years, a new mechanism of energy coupling named flavin-based electron bifurcation (FBEB) has been proposed (8, 9). This mechanism couples a thermodynamically unfavorable reaction (often the reduction of ferredoxin) to a favorable reaction. Reduced ferredoxin (Fd) then functions as an electron and energy carrier and its oxidation can be linked to energy conservation. At the base of FBEB is a stepwise electron transfer from a reduced flavin co-factor (FADH_2) in which the low-potential electron is used for reduction of a high potential electron acceptor, while the second electron reduces Fd (**Figure 1.2**) (9). The reduction of low potential Fd is only possible since a lower redox potential flavin semiquinone (“hot flavosemiquinone”) is formed after the first electron is transferred to the high potential electron acceptor (10). The logic behind FBEB is similar to the Q-cycle proposed by Peter Mitchell, involving a quinone electron bifurcation at the bc_1 complex (11). Energy conservation

via reduced Fd occurs by either proton reduction to H_2 , increasing SLP in the oxidative branch of fermentation, or by generation of an electrochemical gradient via the Rnf membrane complex (8). The Rnf complex (for *Rhodobacter nitrogen fixation*) is a H^+/Na^+ -pumping Fd:NAD $^+$ oxidoreductase found in many anaerobes (12). Since low redox potential Fd is required by autotrophs to reduce CO_2 , FBEB might have been important, if not essential, during early life evolution (13).

1.1.3 – FBEB examples

The FBEB mechanism was first observed in the Gram-positive bacterium *Clostridium kluyveri* (9), which is able to ferment ethanol and acetate to butyrate, caproate, and H_2 , a unique feature among clostridia (14). H_2 formation was shown to be Fd-dependent, requiring acetyl-CoA for reduction of crotonyl-CoA to butyryl-CoA. Also, H_2 was formed by an endergonic reaction during fermentation from NADH. The work of Li *et al.* revealed that the cytoplasmic butyryl-CoA dehydrogenase/electron transfer complex (BcdA/EtfBC complex) was responsible for coupling Fd reduction with NADH to the reduction of crotonyl-CoA to butyryl-CoA with NADH (**Figure 1.2**) (9). Since this complex contains four FAD cofactors with no additional prosthetic groups this new energy conservation mechanism was named flavin-based electron bifurcation. Other examples of FBEB enzymes are the NfnAB complex present in *C. kluyveri*, which couples the reduction of Fd with NADPH to the reduction of NAD $^+$ (15) and the reverse electron bifurcation (confurcation) Hase (HydABC) present in *Thermotoga maritima*, which couples the oxidation of Fd and NADH to generate H_2 (16).

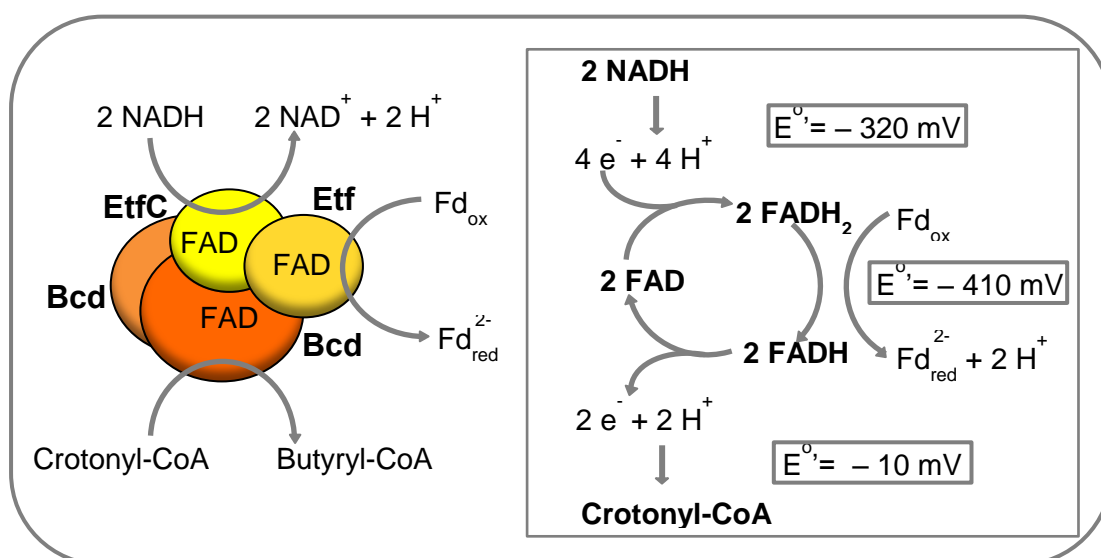


Figure 1.2 – Flavin-based electron bifurcation mechanism by Bcd/EtfCB from *Clostridium kluyveri*. The endergonic reduction of Fd by NADH is coupled to the exergonic reduction of crotonyl-CoA with NADH. Adapted from (9).

In methanogens, the formation of methane from CO₂ reduction with H₂ is coupled to the formation of a heterodisulfide (CoM-S-S-CoB). The mechanism for heterodisulfide reduction is distinct in methanogens with cytochromes from methanogens without cytochromes (17). In methanogens with cytochromes VhoACG hydrogenase/HdrDE heterodisulfide reductase membrane complex couples H₂ oxidation to heterodisulfide reduction and proton pumping (18). In methanogens without cytochromes the MvhADG hydrogenase/HdrABC heterodisulfide reductase cytoplasmic complex couples the reduction of Fd (unfavorable step) with H₂ to the reduction of the heterodisulfide by H₂ (favorable step) in a FBEB mechanism (**Figure 1.3**) (19). Interestingly, we can find HdrABC related proteins in non-methanogenic organisms in which the function of these proteins remains unsolved. The most evident of these Hdr-containing organisms are the sulfate-reducing organisms (SRO) group, whose energy metabolism remains incompletely understood. Surprisingly, these organisms contain several proteins similar to HdrA and HdrB/D (20, 21).

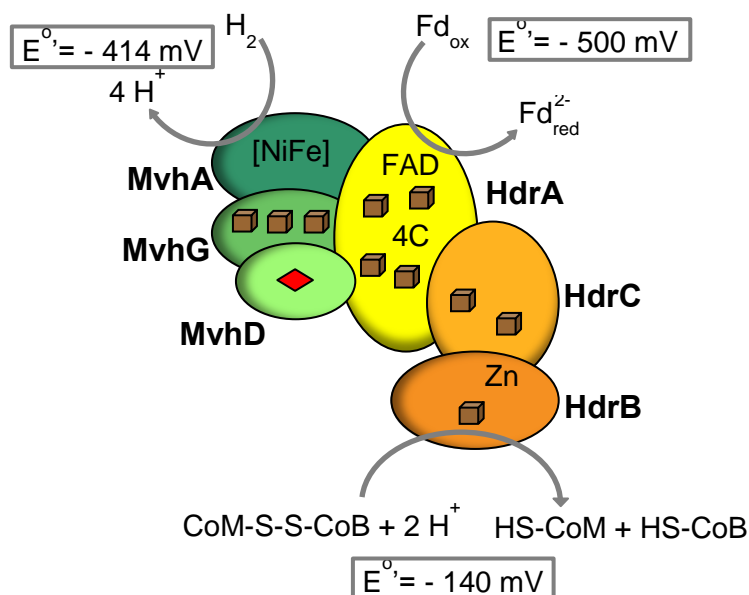


Figure 1.3 – Schematic representation of HdrABC/MvhADG complex from methanogens without cytochromes. The complex is responsible for the endergonic reduction of Fd with H₂ coupled to the exergonic heterodisulfide reduction with H₂. Adapted from (17). C – cysteine; Cube – [4Fe-4S]; diamond – [2Fe-2S].

In the sulfate reducer *Desulfovibrio vulgaris* Hildenborough, a novel gene cluster includes the *floxABCD* (flavin oxidoreductase) genes, which code for a new NADH dehydrogenase and a set of *hdrABC* genes (**Figure 1.4**). The *flox/hdr* cluster is predicted to form an operon possibly involved in electron bifurcation from NADH. Additionally, this cluster is found in a great diversity of Bacteria, including members of different phyla such as *Chlorobi*, *Proteobacteria*, *Firmicutes*, *Bacteroidetes*, *Spirochaetes*, and *Actinobacteria* (20). It is proposed that the Flox proteins oxidize NADH via FloxA that will then transfer electrons to

HdrABC (**Figure 1.5**). In *D. vulgaris* Hildenborough, the *flox/hdr* genes are flanked by two alcohol dehydrogenase genes one of which (*adh1*) is one of the most highly expressed genes in *D. vulgaris* cells, revealing an important function in the energy metabolism (22).



Figure 1.4 – Gene locus of *D. vulgaris* Hildenborough containing the *floxABCD*, *hdrABC*, and *adh* genes (in this organism the *floxC* and *floxD* genes are fused).

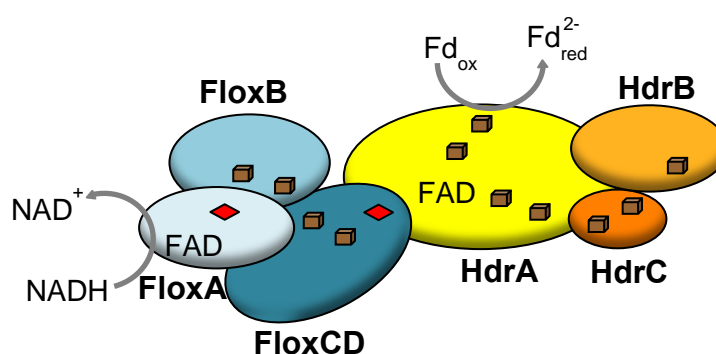


Figure 1.5 – Schematic representation of the *D. vulgaris* Hildenborough Flox and Hdr proteins with corresponding cofactors. NADH may be formed upon oxidation of ethanol by the Adh1 encoded next to the *flox* and *hdr* genes. Cube – [4Fe-4S]; diamond – [2Fe-2S]; Fd – ferredoxin.

1.2 – Sulfate-Reducing Organisms (SRO)

There is geological evidence for the activity of SRO by 3.5 billion years ago (23). Since then, this polyphyletic group of microorganisms has been playing a critical role in the biogeochemical sulfur and carbon cycles. SRO live in anaerobic conditions such as marine sediments, fresh waters, soil and also in the mouth and gut of some animals, including humans. Due to the high concentration of sulfate in sea water (~28 mM) they are more abundant in marine environments and are responsible for 50 % of total carbon mineralization in marine sediments (24). This group of organisms is capable of anaerobic respiration, performing energy conservation through reduction of sulfate to sulfide. Also, since these organisms are able to use a vast variety of compounds as electron donors and electron acceptors, they have a great potential for biotechnological applications, such as bioremediation. Furthermore it was reported that sulfate-reducing bacteria can cause inflammatory bowel diseases in animals and humans (25).

1.2.1 – Sulfur cycle

Sulfur is one of the most abundant elements on Earth and it can be found in different oxidation states, being sulfate (SO_4^{2-} ; oxidation state +6), elemental sulfur (S^0 ; oxidation state 0), and sulfide (S^{2-} ; oxidation state -2) the most significant in nature (26, 27). The sulfur biogeochemical cycle is not only important in geology since many minerals are composed of sulfur but is also essential in biology since amino acids (cysteine and methionine) and many other biomolecules contain sulfur atoms. SRO play a critical role in the sulfur cycle since they use sulfate as terminal electron acceptor in the degradation of organic matter to produce sulfide (**Figure 1.6**). Both these sulfur forms are used in the metabolism of other organisms generating the microbial sulfur cycle (26). Stable isotope fractionation studies show that sulfur has been reduced biologically for over 3.5 billion years (28).

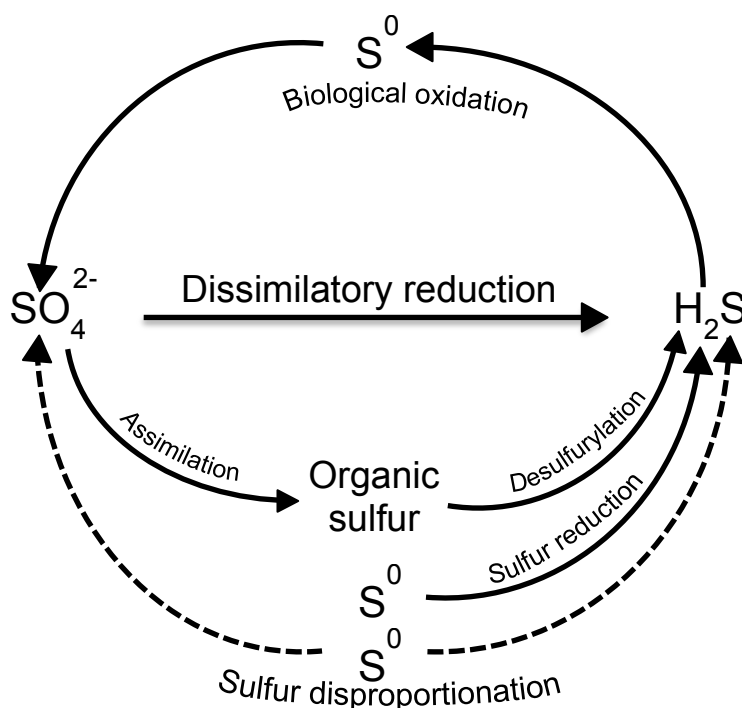


Figure 1.6 – Microbial sulfur cycle. SRO reduce sulfate (SO_4^{2-}) to sulfide (H_2S). Sulfide can then be oxidized aerobically by chemolithotrophic sulfur-oxidizing bacteria (e.g. *Thiobacillus* or *Beggiatoa* spp.) or anaerobically by phototrophic sulfur bacteria (e.g. *Chlorobium* spp.) to elemental sulfur (S^0) and sulfate. Other transformations, which are carried out by specialized groups of microorganisms, result in sulfur reduction (e.g. *Desulfuromonas* spp.) and sulfur disproportionation (e.g. *Desulfovibrio sulfodismutans*). Adapted from (24).

1.2.2 – Physiology and Biochemistry

SRO are chemoheterotrophs since they generate their energy through chemical reactions and require organic carbon for cell growth. They are also capable of either lithotrophic or organotrophic growth since they can use a great diversity of electron donors such as hydrogen, fatty acids, aromatic compounds, sugars, monocarboxylic acids, and dicarboxylic acids (29). Sulfate is not the sole terminal electron acceptor in SRO, as other

sulfur compounds can also be used such as sulfite (SO_3^{2-}), thiosulfate ($\text{S}_2\text{O}_3^{2-}$), elemental sulfur (S^0), and organosulfonates. Moreover, some species of SRO can use other electron acceptors as nitrate and nitrite, which are reduced to ammonium and also metal ion oxides including iron (Fe^{III}), uranyl (U^{VI}), selenite (Se^{VI}), chromate (Cr^{VI}), and arsenate (As^{VI}) therefore revealing a great potential in bioremediation of toxic metals (24).

Many organisms including bacteria, fungi, algae, and plants are capable of assimilatory sulfate reduction in which small amounts of sulfide are produced and incorporated into sulfur-containing amino acids, vitamins and cofactors (30). SRO perform dissimilatory sulfate reduction, *i.e.* the reduction of sulfate for energy conservation while producing large amounts of sulfide, a toxic gas in the protonated form. Moreover, some sulfate reducers are able to survive in low concentrations of oxygen, showing that these organisms are not strict anaerobes (31).

1.2.3 – Taxonomy

The first SRO to be isolated and described was named *Spirillum desulfuricans* due to its morphology and ability to produce sulfide from sulfate (32). This discovery dates from 1895 and was attributed to the Dutch microbiologist and botanist Martinus Beijerinck; the bacterium was later reclassified as *Desulfovibrio desulfuricans* (**Figure 1.7**). Classification of these organisms was based on phenotypic characteristics, which limited the number of species until the 1980's. With the advent of Molecular Biology new methods based on the rRNA 16S gene, PCR, and genome sequencing started to emerge and were used to discover numerous novel species of SRO (24).

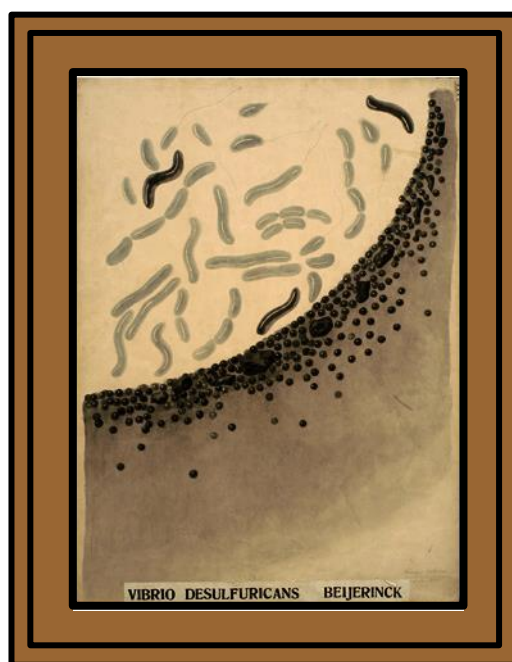


Figure 1.7 – The figure is a reproduction of *Vibrio desulfuricans*, which was painted by Henriëtte Beijerinck, the sister of Martinus Beijerinck, and is reproduced courtesy of the Beijerinck Museum (24).

There are more than 220 species of 60 genera of SRO described, either from the Bacteria or Archaea Domain. Within the Bacteria Domain, these organisms belong to five divisions: *Deltaproteobacteria* class (including *Desulfovibrio* and 11 other genus), *Nitrospira* phylum (containing the genus *Thermodesulfovibrio*), *Clostridia* class (containing the Gram-positive, spore-forming genus *Desulfotomaculum*, *Desulfosporomusa*, and *Desulfosporosinus*), *Thermodesulfobiaceae* family, and *Thermodesulfobacteria* phylum. Within the Archaea Domain, two phyla are distinguished: *Euryarchaeota* (containing the *Archaeoglobus* genus) and *Crenarchaeota* (containing the genus *Thermocladium* and *Caldivirga*) (**Figure 1.8**) (24, 29). From these organisms, 72 genome sequences are already available according to the Integrated Microbial Genomes (IMG) website.

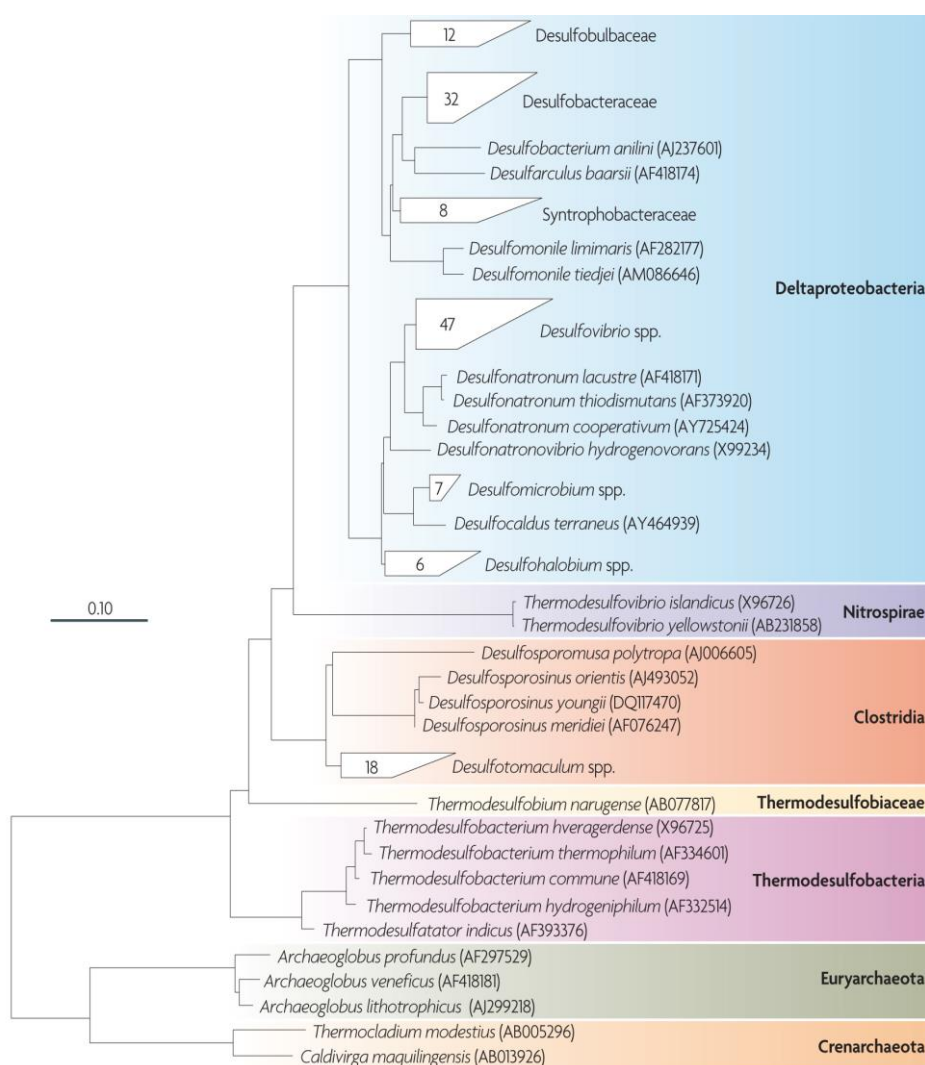


Figure 1.8 – Phylogenetic tree of the SRO described, based on 16S ribosomal RNA (rRNA) sequences. It is possible to observe five phylogenetic lineages of Bacteria Domain organisms and two of Archaea Domain organisms. The 0.10 scale bar indicates 10 % of sequence difference. From (24).

1.2.4 – Environmental impact

SRO have an important environmental impact since they intervene in biocorrosion of ferrous metals and also corrosion of concrete and stonework (29). These microorganisms also have a negative impact on the petroleum industry since they can contribute to a phenomenon called “oil souring” by sulfide production in oil fields and pipelines.

Since SRO have a very plastic metabolism they have a great biotechnological potential. These microorganisms are capable of bioremediation of several compounds by oxidation, such as organic compounds, monoaromatic hydrocarbons present in contamination due to petroleum spills; reduction of halogenated compounds, which contaminate waters and sediment, and are used as terminal electron acceptor (dehalorespiration); nitroaromatic respiration, such as TNT (trinitrotoluene) which contaminates soils and ground water near places where explosives are manufactured (29). Also, SRO are capable of immobilization of hazardous and toxic metals released by metallurgic plants, nuclear plants or oil refining industry via precipitation as metal sulfides. A restricted group of SRO called DMRB (dissimilatory metal-reducing bacteria) is capable of toxic metal reduction (such as uranium) posing an important contribution for toxic environment bioremediation. Reduction of azo dyes, which contain an azo bond ($N=N$) and are abundantly produced in the textile industry, is also performed by this group of organisms; finally, SRO are capable of biorecovery of precious metals such as gold, platinum, or palladium from industrial waste streams (33).

Concerning the high potential of SRO in biotechnology, the study of the physiology and full comprehension of the metabolism of these organisms is imperative to further develop new and more powerful biotechnological tools.

1.3 – The *Desulfovibrio* genus

Desulfovibrio is one of many genus of sulfate-reducing bacteria belonging to the *Deltaproteobacteria* class. *Desulfovibrio* species exist in marine environments, but also in hot springs, geothermal vents, the human digestive tract, and in soil (34). These bacteria are not strict anaerobes as previously considered, since they tolerate oxygen concentration but their growth is limited in aerobic environments. The optimal growth temperature for the mesophilic *Desulfovibrio* bacteria ranges from 25 – 40 °C while the optimal pH ranges between 6.6 – 7.5. Since *D. vulgaris* Hildenborough was the first SRO to have its genome sequenced and due to its easy and rapid growth it has been used as a model for studying the physiology, genetics, and biochemistry of these organisms (35).

1.3.1 – Morphology, biochemistry, and genome features

Desulfovibrio species are non-sporulating curved rod-shaped cells with sizes between $0.5 - 1.3 \times 0.8 - 5 \mu\text{m}$ (36). These cells are motile with a single polar flagellum and stain Gram negative. These microbes use hydrogen, organic acids, or alcohols as electron donors for sulfate reduction. Lactate is preferentially used for carbon metabolism but pyruvate, formate, and certain primary alcohols also function as carbon sources. The model organism *D. vulgaris* Hildenborough was discovered and isolated in 1946 in clay soil near Hildenborough, Kent, England (35). The genome contains two replicons: one chromosome containing 3.57 Mbp and one mega plasmid containing 202 kbp. The GC percentage of the replicons is 63.2 and 65.7, respectively (35).

1.3.2 – Taxonomy

D. vulgaris Hildenborough belongs to the phylum *Proteobacteria* and class *Deltaproteobacteria*. The order of these bacteria is *Desulfovibrionales*, the family is *Desulfovibrionaceae* and the genus is *Desulfovibrio*. In the last decade several genomes of SRO were sequenced and deposited in online databases such as MicrobesOnline, Integrated Microbial Genomes (IMG), and National Centre for Biotechnology Information (NCBI). There are over 60 known species of the *Desulfovibrio* genus, from which 43 genomes are available according to the IMG website. Very recently, the genome of *Desulfovibrio gigas* was sequenced through a collaboration between a group at ITQB and Brazilian researchers (37).

1.3.3 – Dissimilatory sulfate reduction

Dissimilatory sulfate reduction occurs in the cytoplasm of SRO and requires the presence of four soluble enzymes. In order to fully reduce sulfate to sulfide, eight electrons are necessary and approximately two ATP molecules are used for sulfate activation (**Figure 1.9**). Sulfate is transported inside the cell by symport with Na^+ or H^+ , depending on the marine or fresh-water environment, respectively (38). Since sulfate reduction to sulfite is chemically unfavorable [$E^\circ (\text{SO}_4^{2-}/\text{SO}_3^{2-}) = -516 \text{ mV}$] sulfate requires activation to a more favorable compound. In the presence of the ATP sulfurylase or adenylyltransferase (Sat), sulfate and ATP are converted to adenosine-5'-phosphosulfate (APS generating pyrophosphate (PP_i)). Some SRO have membrane associated pyrophosphatases that couple the hydrolysis of PP_i to proton translocation across the membrane minimizing the energy costs of sulfate reduction (20). APS [$E^\circ (\text{APS}/\text{SO}_3^{2-}) = -60 \text{ mV}$] accepts two electrons being reduced to sulfite and AMP by the APS reductase enzyme (AprBA). The physiological electron donor to AprBA is still unknown but several lines of evidence point to the Quinone-interacting membrane-bound oxidoreductase (QmoABC) membrane complex (39, 40). After sulfite formation, the final reduction of sulfite to sulfide [$E^\circ (\text{SO}_3^{2-}/\text{H}_2\text{S}) = -116 \text{ mV}$] requires 6 electrons and two mechanisms were proposed to explain this reduction: the trithionate pathway, in which sulfite is reduced to sulfide in three steps, by the intermediates trithionate ($\text{S}_3\text{O}_6^{2-}$) and thiosulfate ($\text{S}_2\text{O}_3^{2-}$) (32); and the second mechanism, which explains the 6 electron reduction in only one

reduction. However, DsrD was not found associated with DsrAB during the purification methods and *in vitro* studies revealed that this protein binds sulfate, sulfite, and sulfide with low affinity (47). Since the structure of DsrD includes a winged-helix motif, a B- and Z-DNA binding motif, it has been hypothesized that DsrD is associated to sulfate reduction not in a direct biochemical association but via regulation of sulfite reduction genes (**Figure 1.10**) (48). Despite some structural, biophysical, and biochemical studies performed with this protein, the physiological function of DsrD remains to be solved.

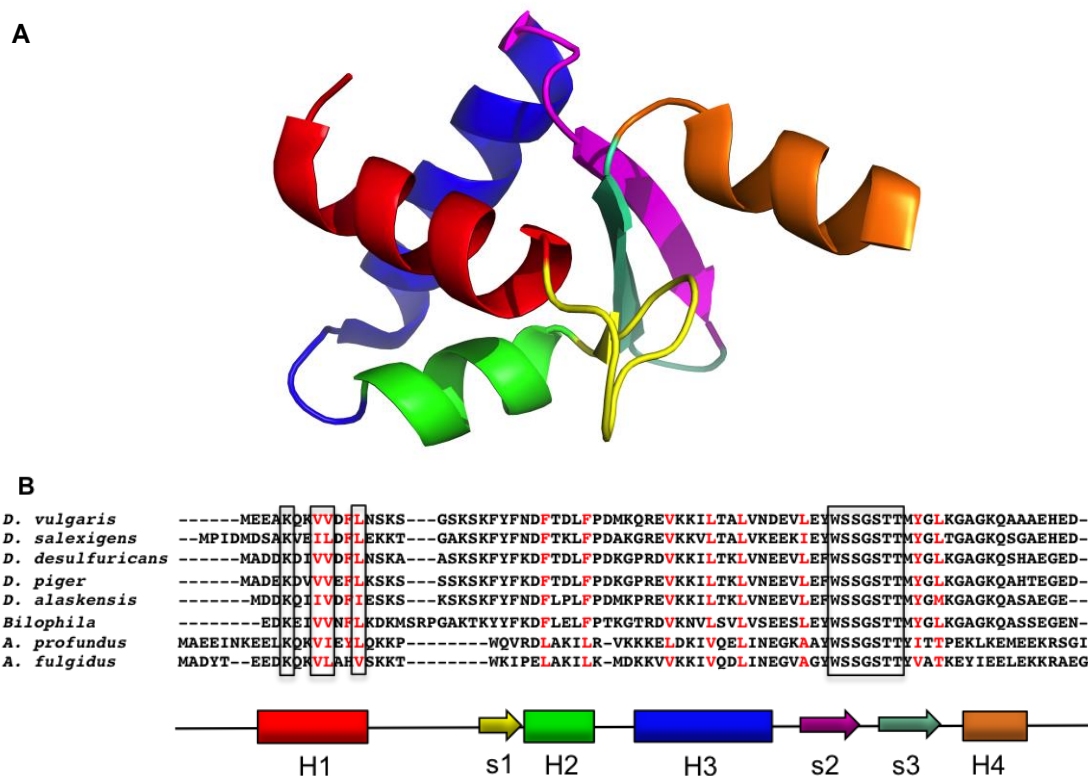


Figure 1.10 – (A) Stereo view of DsrD from *D. vulgaris* Hildenborough at 1.2 Å resolution. DsrD has a winged-helix motif composed of three α -helices (H1, H2, H3) and three β -sheets (s1, s2, s3). DsrD has an additional α -helix (H4) in the C-terminal region (B) Sequence alignment of DsrD from *D. vulgaris* Hildenborough, *D. salexigens*, *D. desulfuricans*, *D. piger*, *D. alaskensis*, the DsrD fragment (residues 405-483 of DsrBD) from *Bilophila wadsworthia*, DsrD from *Archaeoglobus profundus*, and *Archaeoglobus fulgidus*. Box – highly conserved residues in the DsrD family; red – highly conserved hydrophobic residues in the winged-helix motif. Adapted from (48).

2 – Objectives

This thesis focuses on two main objectives.

The first objective is to study the *hdr-flox* gene cluster possibly involved in an electron bifurcation pathway present in the model organism *D. vulgaris* Hildenborough. It was ultimately desired to elucidate and better understand the metabolic importance of this gene cluster and to analyze in which conditions these genes were more expressed both at a gene and protein level.

The second part of this thesis focuses on the study of *dsrD*, a potentially relevant gene involved in the dissimilatory sulfate reduction whose function in the physiology of SRO remains a mystery. The main objective of this study is to provide new hints about the function of this protein both with *in vitro* and *in vivo* assays, while contributing to a better understanding of the dissimilatory sulfate reduction pathway, in particular in the sulfite reduction.

3 – Materials and Methods

3.1 – *hdr-flox* gene cluster

3.1.1 – Strains and media

The strains used in this work were previously constructed by Fabian Grein (49) and are listed in **Table 3.1**. All *D. vulgaris* Hildenborough strains were grown in anaerobic conditions, in MOY basal medium (39). MOY medium contains 8 mM MgCl₂, 20 mM NH₄Cl, 0.6 mM CaCl₂, 2 mM K₂HPO₄-NaH₂PO₄, 0.06 mM FeCl, 0.12 mM EDTA, 30 mM Tris-HCl pH 7.4, 1 g/L of yeast extract, 6 mL of trace element solution (39), and 1 mL of Thauers vitamins solution (50) per liter. Thauer's vitamin solution contains 82 µM biotin, 45 µM folic acid, 468 µM pyridoxine hydrochloride, 148 µM thiamine hydrochloride, 133 µM riboflavin, 406 µM nicotinic acid, 210 µM DL-panthotenic acid, 365 µM *p*-aminobenzoic acid, 242 µM lipoic acid, 14 mM choline chloride, and 7.4 µM vitamin B12. Additionally, 1.2 mM thioglycolate was used as a reducing agent and 640 nM resazurin solution was used as a redox potential indicator (which becomes pink when the potential exceeds 110 mV). The pH was adjusted with 1 M HCl to the final value of 7.2. MOY was supplemented with different electron donors-acceptors in five different culture conditions: 60 mM lactate-30 mM sulfate (LS4); 30 mM lactate-20 mM sulfite (LS3); 60 mM pyruvate-2 mM sulfate (P); 40 mM ethanol-20 mM sulfate (ES4); and finally 1 bar hydrogen (H₂)-30 mM sulfate supplemented with 10 mM acetate (HS4). The cultures containing hydrogen as electron donor were gassed at 1 atm pressure with 80% H₂ (v/v) and 20 % CO₂ and incubated at 37 °C in a horizontal position in order to increase the gas-liquid surface area.

3.1.2 – Growth curves

D. vulgaris Hildenborough WT and mutant strain cultures were grown anaerobically at 37 °C in 100 mL flasks containing a final volume of 50 mL. All flasks were inoculated with 2 % (v/v) of fresh precultured cells grown on lactate-sulfate medium except for the pyruvate growth (P), in which cells were precultured in pyruvate medium (P), and for the media with hydrogen, in which 10 % (v/v) of pre-culture cells was used. The optical density (OD at 600 nm) was monitored at various time points with a spectrophotometer Shimadzu UV-1603. All reported optical density measurements are the mean of four biologically independent experiments.

3.1.3 – Ethanol quantification

Ethanol accumulation in the growth medium was determined with an enzymatic kit from NZYTech. This method is based on quantifying NADH formed from ethanol through the combined action of Alcohol dehydrogenase (Adh) and aldehyde dehydrogenase (Al-dh).

Table 3.1 – List of strains, plasmids, and primers used in this work.

Strain	Genotype	Source and/or reference
<i>E. coli</i> α-Select Bronze efficiency	F^- <i>deoR endA1 relA1 gyrA96 hsdR17(r_k⁻ m_k⁺) supE44 thi-1</i> $\Delta(lacZYA argFV169) \Phi 80\delta lacZ \Delta M15 \lambda$	Bioline
<i>E. coli</i> BL21 (DE3) Gold	<i>E. coli</i> B F^- <i>dcm+ Hte ompT hsdS(r_B - m_B-) gal λ (DE3) endA Tet^r</i>	Stratagene
ATCC 29579	<i>Desulfovibrio vulgaris</i> Hildenborough WT	ATCC
IPFG01	WT <i>hdrC::ΩKm</i>	(Ramos <i>et al.</i> 2014)
IPFG02	WT $\Delta floxA::Km^R$	(Ramos <i>et al.</i> 2014)
IPFG03	WT $\Delta floxA::Km^R$ + pMOIP12P	(Ramos <i>et al.</i> 2014)
IPFG04	WT <i>floxA</i> -Strep tag	(Ramos <i>et al.</i> 2014)
<i>dsrD</i> mutant	WT $\Delta dsrD::Km^R$	This study
Plasmid	Relevant characteristics	Source and/or reference
pMO719	pCR8/GW/TOPO containing SRB replicon (pBG1); Spec ^R	(Keller <i>et al.</i> 2009)
pSC27	<i>Desulfovibrio</i> shuttle vector; source of <i>aph</i> (3')-II; Km ^R	(Rousset <i>et al.</i> 1998)
pMOIP17	<i>dsrD</i> deletion plasmid, Spec ^R , Km ^R	This study
pMOIP12P	<i>floxA</i> from IBAFloxASTrep in pMOIP05 (replacing Insert using <i>NdeI</i> and <i>EcoRI</i>) for plasmid encoded tagging, Spec ^R	(Ramos <i>et al.</i> 2014)
pET ₂₂ b(+)	Bacterial protein expression vector; <i>peB</i> sequence for periplasmic localization; C-terminal His-tag; Amp ^R ; lac operator	Novagen
pET ₂₂ <i>dsrD</i>	DsrDrec expression vector	This study
Primer	Details	Sequence (5'-3')
<i>dsrD</i> fw	<i>dsrD</i> gene DVH forward with <i>NdeI</i> restriction site	GGGGTACTCATATGGAAGAAGCC
<i>dsrD</i> rev	<i>dsrD</i> gene DVH reverse with <i>XhoI</i> restriction site	TGAAGGCTCGAGTTCGTGCTC
T7 promoter	pET ₂₂ b(+) T7 promoter region	TAATACGACTCACTATAGGGG
T7 terminator	pET ₂₂ b(+) T7 terminator region	GCTAGTTATTGCTCAGCGG
Primer 97	<i>dsrD</i> upstream forward	GCCTTTTGCTGGCCTTTTGCTCA CATATGTCGATCACCCACATCCG
Primer 98	<i>dsrD</i> upstream reverse	AAGACTGTAGCCGTACCTCGAAT CTATTGCTCTTGCTACCAGACTTGG
Primer 99	<i>dsrD</i> downstream forward	AATCCGCTCACTAAGTTCATAGA CCGAGCCAGGAAGCGTATGAACC
Primer 100	<i>dsrD</i> downstream reverse	CGAGGCATTCTGTCTGGCTGG CTGACGAAGGGCTTCAAGGT
Primer 9	Km ^R forward	TAGATTCGAGGTACGGCTACAGTCTTAC GGTCACAAACAGGTACGCCCCAGAGTC CCGCTCAG
Primer 10	Km ^R reverse	CGGTCTATGAACTTAGTGAGCGGATTTCT CGTGTAGCCGATGCAGTGAGGTAGCTTG CAGTGGGCT
Primer 11	Spec ^R forward	CCAGCCAGGACAGAAATGCCTCG
Primer 12	Spec ^R reverse	ATGTGAGCAAAAGGCCAGCAAAAGGC

3.1.4 – Adh1 purification and activity measurements

Adh1 was purified inside a Coy anaerobic chamber [95 %/ 5 % (v/v) N₂/H₂] using an AKTA™ Prime plus™ system from the soluble fraction of IPFG04 cells grown in ethanol, using first a Q-Sepharose HP according to (51). NaCl was added to the fraction with Adh activity, to a concentration of 1 M. The fraction was then purified in a HiTrap™ Phenyl HP (2 mL column volume, CV) equilibrated with 50 mM Tris-HCl (pH 7.6) and 1 M NaCl. The protein elution was performed with a linear gradient of 1 to 0 M NaCl (20 CV). Fractions with highest Adh activity eluted between 1 and 0.7 M NaCl. The identity of the enzyme purified was confirmed as Adh1 by Mass spectrometry. Adh activity was determined by NADH formation at 340 nm ($\epsilon_{\text{NADH}} = 6.22 \text{ mM}^{-1} \text{ cm}^{-1}$) in 50 mM Tris-HCl (pH 9), 5 mM NAD⁺, and 20 mM ethanol, at room temperature (51) with a Shimadzu UV-1800 spectrophotometer in a stirred cuvette (Hellma). Adh1 antibody was produced from pure protein injected in rabbits, at Davids Biotechnologie GmbH®.

3.1.5 – Reverse Transcriptase quantitative PCR (RT-qPCR)

Reverse Transcriptase quantitative PCR (RT-qPCR) was used in *D. vulgaris* WT to analyze gene expression of *adh1*, *floxA*, and *hdrA* in cells grown in LS4, LS3, P, ES4, and HS4. Cells from three independent experiments were collected at the mid-exponential phase, centrifuged for 12 min at 3,000 *g*, washed with ice-cold sterile Mili-Q water, and frozen for later RNA extraction. Total RNA was extracted as described in (52). DNase treatment was performed with Turbo DNase (Ambion) in order to avoid genomic DNA contamination in the RNA extracts and was also followed by a RNA clean-up kit (Qiagen). cDNA synthesis from each RNA sample (1 µg) was performed using Transcriptor Reverse Transcriptase (Roche Diagnostics). Primers were designed to amplify a region of about 100 bp of *adh1*, *floxA*, *hdrA*, and the 16S rRNA gene was used as an internal reference gene for each analyzed sample (Table 3.2). Reverse Transcriptase quantitative PCR reactions were performed in a Light Cycler 480 Real-Time PCR System (Roche), with Light Cycler 480 SYBR Green Master I (Roche). Relative standard curves and gene expression were calculated by the relative quantification method with efficiency correction, using the LightCycler Software 4.1, using 16S rRNA gene as a reference (53). For the final results three biological replicates and two technical replicates were used for each condition. The unpaired (two-sample equal variance with two-tailed distribution) *t*-test was used to determine the significance of the differences in gene expression between cells grown in LS4 and cells grown in the other culture conditions, with a significance defined as *p*-value < 0.10 and *p*-value < 0.05.

Table 3.2 – Primers used in qPCR to study the expression of *adh1*, *floxA*, and *hdrA*.

Target	Primer sequence (5' → 3')	Amplicon size (bp)
<i>adh1</i>	Forward: ACCAAGAACGCGCAGAA Reverse: CGGTTCTGTCTGTACTCCTTAC	111
<i>floxA</i>	Forward: ACCAAGTACGTGTGTGTCG Reverse: CTGCATCGCGGCTACAA	83
<i>hdrA</i>	Forward: CATTCCCAAGAAGGCGATCA Reverse: CGACAATCTCATCTCCATGTC	125
16S rRNA gene	Forward: CCTATTGCCAGTTGCTACC Reverse: AAGGGCCATGATGACTTGAC	100

3.1.6 – Western blot analysis of FloxA, HdrA, and Adh1 expression

D. vulgaris WT cells grown in lactate-sulfate (LS4), lactate-sulfite (LS3), pyruvate (P), ethanol-sulfate (ES4), and hydrogen-sulfate (HS4), were collected at two different time points, mid-exponential and stationary phase, and centrifuged for 12 min at 3,000 g. Cells were then disrupted by adding 1 mL of BugBuster® Protein Extraction Reagent (Novagen®) and 10 µL of lysonase per g of cells, followed by 20 min incubation with slow stirring at room temperature. The soluble crude extract was obtained by centrifugation at 16,000 g and 4 °C for 20 min. Protein concentration was determined by the Bradford method (Bio-Rad) with bovine serum albumin as standard (NZYTech).

Protein samples of 25 µg were run in a SDS-PAGE gel [12 % acrylamide, (v/v)] and transferred to 0.45 µm polyvinylidene difluoride (PVDF) membranes (Roche) for 30 minutes at 100 V and 350 mA in a Mini Trans-Blot® electrophoretic transfer cell (Bio-Rad) containing Transfer Buffer (48 mM Tris pH 9.2, 39 mM glycine). The membranes were treated with Blocking Buffer [20 mM Tris-HCl pH 7.5, 150 mM NaCl, 0.05 % Tween 20 (v/v), and 5 % non-fat milk (w/v)], overnight at room temperature. In the following day, after three washing steps with TBST [20 mM Tris-HCl pH 7.5, 150 mM NaCl, 0.05 % Tween 20 (v/v)], anti-FloxA at 1 : 1000, anti-HdrA 1 : 500, and anti-Adh1 at 1 : 5000 dilution in TBST were incubated with the membrane for 1 h at room temperature; after two washing steps with TBST, membranes were incubated with anti-rabbit IgG antibody linked to alkaline phosphatase conjugate (Sigma-Aldrich®) at 1 : 15 000 dilution in TBST for 45 minutes. After three washing steps with TBS (20 mM Tris-HCl pH 7.5, 150 mM NaCl) protein detection was performed with Alkaline Phosphatase Buffer (100 mM Tris-HCl pH 9.5, 100 mM NaCl, and 5 mM MgCl₂) and NBT (nitro-blue tetrazolium chloride)/BCIP (5-bromo-4-chloro-3-indolyl phosphate) (Carl Roth®). The antibodies used against FloxA and HdrA subunits were produced in rabbits with synthesized peptides and Adh1 antibody was produced from pure protein, as previously referred.

3.2 – Studies with *dsrD*

3.2.1 – *dsrD* heterologous expression

3.2.1.1 – *dsrD* gene cloning and protein expression

The *dsrD* gene of *Desulfovibrio vulgaris* Hildenborough was amplified by PCR using genomic DNA as template and the *dsrD* fw and *dsrD* rev primers with *Nde*I and *Xho*I restriction sites, respectively (**Table 3.1**). The PCR product was cloned into a pET_{22b}(+) vector (Novagen®), allowing the insertion of a 6-His tag at the C-terminus of DsrD protein. The recombinant plasmid (pET₂₂dsrD) was heat-shock transformed into *E. coli* BL21 (DE3) Gold (Stratagene®) strains which were grown at 37 °C in Minimal Medium with ampicillin (100 µg/mL) until an optical density of 0.4. Then 100 µM IPTG (isopropyl β-D-1-thiogalactopyranoside) was added and cells were permitted to grow for 4 additional hours.

3.2.1.2 – DsrD purification

The cells were harvested by centrifugation, washed with Buffer A (25 mM potassium phosphate, 300 mM NaCl, 30 mM imidazole pH 7.2) and frozen to – 20 °C. After thawing and resuspending with Buffer A the cells were disrupted using a French Press cell in the presence of DNase. Afterwards, the cell lysate was centrifuged for 15 minutes at 20,000 *g* in order to remove cell debris, and then ultra centrifuged for 2 h at 140,000 *g* to separate the membrane from the soluble fraction. The supernatant was injected into a HiTrap Chelating HP column (GEHealthcare) charged with NiCl₂ and equilibrated with Buffer A. The protein was eluted with Buffer B (Buffer A with 100 mM imidazole) and dialyzed to 25 mM potassium phosphate pH 7. The protein purity was analyzed by SDS-PAGE stained with Coomassie Blue and the concentration was determined at 280 nm using the absorption coefficient of 10 mM⁻¹cm⁻¹ and with the BCA (bicinchoninic acid assay) kit by Pierce®.

3.2.1.3 – DsrD activity and interaction assays

DsrABC was tested for sulfite reduction activity in the presence of DsrD in order to analyze if there was any interaction between these molecules capable of modifying the reaction rate. The enzymatic activities were performed in a Coy anaerobic chamber [95 %/ 5 % (v/v) N₂/H₂] with a Shimadzu UV-1800 spectrophotometer in a stirred cuvette (Hellma), monitoring the oxidation of methyl viologen (an electron acceptor reduced by Zn²⁺) mediated by DsrABC, which transfers electrons to sulfite. The assays were developed in Wash Buffer (25 mM KPi pH7) at room temperature, measuring the variation of Abs_{732 nm} through time.

BIACore experiments were performed with DsrD and DsrABC. BIACore is a technology based on the Surface Plasmon Resonance (SPR) principle, which permits the determination of affinity constants between two interacting proteins. DsrD was chemically immobilized in a CM5 sensor chip (GE® Healthcare) to which DsrABC was injected and interaction of these two proteins was measured. The SPR experiments were performed at 25 °C on a BIACore 2000 instrument (Biacore Inc., GE® HealthCare).

Additionally, Isothermal Titration Calorimetry (ITC) technique was also performed to measure interactions between DsrD and DsrABC or sulfite. ITC is a biophysical method that allows the measurement of thermodynamics and binding association of molecules in solution by detecting the heat change in molecular reactions. The ITC experiments were performed at 25 °C on a MicroCal™ (GE® Healthcare).

3.2.2 – *dsrD* deletion

3.2.2.1 – Strains and media

The strains used in this work are listed in **Table 3.1**. *Escherichia coli* α -Select strain was cultured in LB medium (per liter of medium: 10 g tryptone, 10 g sodium chloride, and 5 g yeast extract). Where indicated, kanamycin or spectinomycin was added to LB medium to a final concentration of 50 μ g/mL and 100 μ g/mL, respectively. All *D. vulgaris* strains were grown at 37 °C in MOY medium according to (39). Sodium lactate (60 mM) or sodium pyruvate (30 mM) were added as electron donors and sodium sulfate (30 mM for lactate and 3 mM for pyruvate) or sodium sulfite (20 mM for lactate) were added as terminal electron acceptors. Antibiotics were added to the MOY medium as follows: G418 (geneticin) at 400 μ g/mL or spectinomycin at 100 μ g/mL. G418 was used in place of kanamycin as described by Zane *et al.* 2010. For solidified MOY medium, 15 g agar per liter was added.

3.2.2.2 – Plasmid and strain construction

The pMOIP17 plasmid for insertion of Ω Km cassette in *dsrD* was constructed by SLIC (sequence ligation independent cloning). The upstream and downstream regions flanking *dsrD* were amplified using the primers 97, 98, 99, and 100, respectively, (**Table 3.1**) and chromosomal DNA of *D. vulgaris* was used as template. Also, a spectinomycin resistance cassette and the pMO719 template containing pUC ori and Kanamycin resistance cassette were all amplified by PCR, using pSC27 and pMO719, respectively, as templates and primers 9, 10, 11, and 12. The four PCR products were added to a reaction mix with T4 ligase incubated for 30 min at room temperature. 1 mM dCTP (deoxycytidine triphosphate) was then added the DNA was heat-shock transformed into *E. coli* α -Select competent cells and plated into kanamycin and spectinomycin containing LA plates. The assembled plasmid (pMOIP17) was extracted, sequenced, and electroporated into *D. vulgaris* cells according to Keller *et al.* (2011) from which double recombinants were selected in MOY solid medium with 30 mM of sodium pyruvate by secondary antibiotic screening as described in (54). G418 was used in place of kanamycin as described by Zane *et al.* (2010). G418 resistant but spectinomycin sensitive colonies were selected and allowed to grow in G418 containing growth medium. The absence of the *dsrD* gene in the *dsrD* mutant strain was verified by PCR using the *dsrD* fw and *dsrD* rev primers with the chromosomal DNA extracted from the screened cells.

3.2.2.3 – Growth curves

D. vulgaris Hildenborough WT and *dsrD* mutant strains were grown anaerobically at 37 °C in 100 mL flasks with 50 mL of MOY basal medium (39). MOY was supplemented with different electron donors-acceptors in three different culture conditions: 60 mM lactate-30 mM sulfate (LS4); 30 mM lactate-20 mM sulfite (LS3); 30 mM pyruvate-3 mM sulfate (Pyr). All media were inoculated with 2 % (v/v) fresh precultured cells grown in pyruvate-sulfate medium (Pyr). The OD of the cultures was monitored at various time points with a spectrophotometer Shimadzu UV-1603. All reported optical density measurements are the mean of three biologically independent experiments.

4 - Results

4.1 – The *hdr-flox* gene cluster

4.1.1 – Growth curves

To evaluate the physiological function of the *flox* and *hdr* genes, the strains IPFG01 (*hdrC*:: Ω Km) and IPFG02 (Δ *floxA*::Km^R) were generated where a kanamycin resistance cassette was inserted in the *hdrC* gene, preventing translation of downstream genes (IPFG01), and the *floxA* gene was specifically deleted by replacement with a kanamycin resistance cassette (IPFG02). Additionally, in order to confirm that the phenotype of IPFG02 was due to the absence of *floxA*, a FloxA-complemented strain expressing FloxA from a plasmid was constructed and named strain IPFG03 (IPFG02 + pMOIP12P). *D. vulgaris* WT, IPFG01, and IPFG02 strains were grown in lactate-sulfate (LS4), lactate-sulfite (LS3), pyruvate fermentation (P), ethanol-sulfate (ES4), or hydrogen-sulfate (HS4) medium (**Figure 4.1, Table 4.1**). The three mutant strains behaved similarly to WT using lactate as an electron donor, both with sulfate or sulfite as terminal electron acceptors (**Figure 4.1A, 4.1B**), whereas in pyruvate fermentation strains IPFG01 and IPFG02 had a similar growth rate, but the final OD was slightly lower than the WT and complemented strains (**Figure 4.1C**). Interestingly, in ethanol-sulfate conditions both mutant strains, IPFG01 or IPFG02, were unable to grow, while the complemented strain grew to a similar growth rate and maximum OD compared to the WT (**Figure 4.1D**). In HS4 medium strains IPFG01 and IPFG02 grew somewhat slower than the WT and reached a lower cell density. Since IPFG02 was not capable of growing in ES4 medium while WT and IPFG03 did, we can conclude that FloxA is essential for ethanol oxidation in *D. vulgaris*. Ethanol has also been reported as a metabolic product of *D. vulgaris* Hildenborough, produced in higher amounts with pyruvate as an electron donor than with lactate (55). To check whether the Hdr-Flox proteins are involved in the production of ethanol as a fermentative product during growth on pyruvate, we quantified ethanol accumulated in the growth media of the WT and mutant strains. The WT strain accumulated a considerable amount of ethanol in the growth medium, while ethanol was absent in the two mutant strains IPFG01 and IPFG02 (**Figure 4.2**). The complemented strain IPFG03 produced similar levels of ethanol as the WT, confirming a role of Flox-Hdr in ethanol production during pyruvate fermentation.

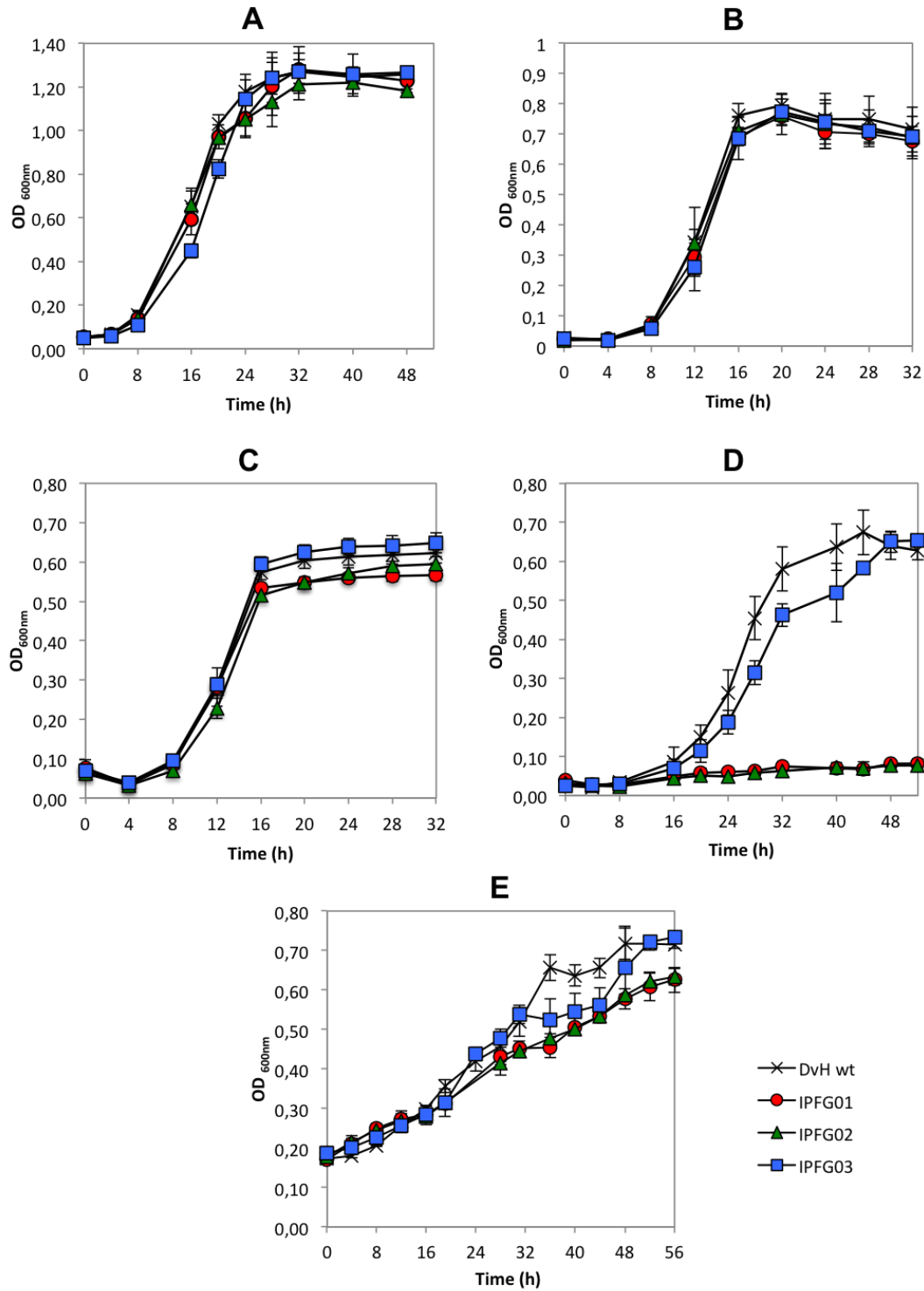


Figure 4.1 – Growth curves of *D. vulgaris* WT and mutant strains in (A) lactate-sulfate, (B) lactate-sulfite (C) pyruvate fermentation, (D) ethanol-sulfate, and (E) hydrogen-sulfate conditions. The points are means of four independent growth experiments, and error bars give standard deviations. IPFG01 – *hdrC::ΩKm*; IPFG02 – Δ *floxA::Km^R*; IPFG03 – Δ *floxA::Km^R* + pMOIP12 (complemented strain).

Table 4.1 – Specific growth rate (μ_g), doubling time (T_d), and maximal OD (600 nm) for *D. vulgaris* and mutant strains in different conditions: lactate-sulfate (LS4), lactate-sulfite (LS3), pyruvate (P), ethanol-sulfate (ES4), and hydrogen-sulfate (HS4). IPFG01 – *hdrC::ΩKm*; IPFG02 – Δ *floxA::Km^R*; IPFG03 – Δ *floxA::Km^R* + pMOIP12.

Medium	LS4			LS3			P			ES4			HS4		
Strain	μ_g (h ⁻¹)	T_d (h)	Max. OD ₆₀₀	μ_g (h ⁻¹)	T_d (h)	Max. OD ₆₀₀	μ_g (h ⁻¹)	T_d (h)	Max. OD ₆₀₀	μ_g (h ⁻¹)	T_d (h)	Max. OD ₆₀₀	μ_g (h ⁻¹)	T_d (h)	Max. OD ₆₀₀
WT	0.174	4.0	1.257	0.315	2.2	0.715	0.230	3.0	0.623	0.119	5.8	0.627	0.040	17.4	0.715
IPFG01	0.169	4.1	1.228	0.290	2.4	0.676	0.237	2.9	0.567	0	0	0.083	0.025	28.0	0.626
IPFG02	0.172	4.0	1.184	0.300	2.3	0.689	0.239	2.9	0.596	0	0	0.078	0.026	27.0	0.633
IPFG03	0.150	4.6	1.267	0.305	2.3	0.690	0.232	3.0	0.649	0.119	5.8	0.654	0.038	18.3	0.733

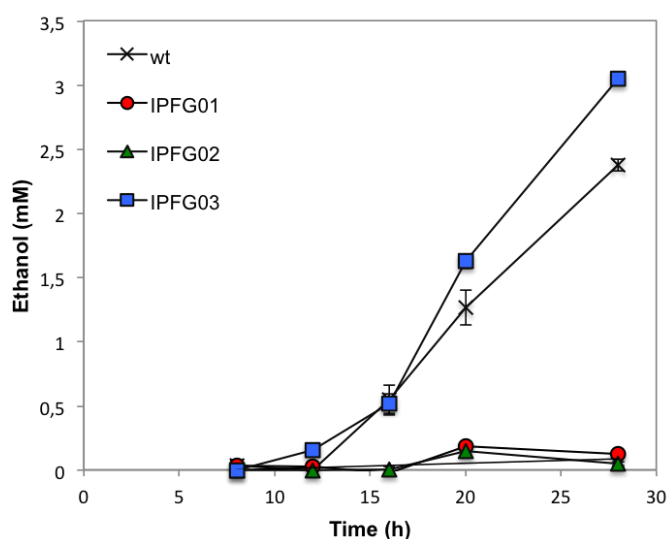


Figure 4.2 – Ethanol (mM) accumulated during growth of *D. vulgaris* WT and mutant strains in pyruvate fermentation conditions (P). IPFG01 – *hdrC::ΩKm*; IPFG02 – Δ *floxA::Km^R*; IPFG03 – Δ *floxA::Km^R* + pMOIP12 (complemented strain).

4.1.2 – Adh1 purification and activity measurements

To test whether the FloxABCD-HdrABC complex is involved in electron bifurcation it is essential to isolate the proteins and perform the bifurcation assays *in vitro*. Several attempts were made to purify the Hdr and Flox proteins but due to their very low expression it was not possible to obtain these proteins in the amount required for enzymatic assays. However, Adh1 was purified without difficulty to a specific activity of 4.6 U/mg, confirming that this protein is abundant in *D. vulgaris*. We confirmed its co-enzyme preference for NAD⁺, which gave a 10-fold higher activity than NADP⁺.

4.1.3 – Reverse Transcriptase quantitative PCR (RT-qPCR)

In order to get more insight into the physiological function of the *hdr-flox* gene cluster we studied the relative gene expression of *floxA*, *hdrA*, and *adh1* genes by RT-qPCR at mid-exponential growth phase from cells grown with lactate, ethanol, or hydrogen as electron donors for sulfate reduction, by pyruvate fermentation, and with lactate as electron donor and sulfite as electron acceptor (**Figure 4.3**). The *hdrA* gene shows the highest expression in ethanol-sulfate (ES4) followed by pyruvate fermentation (P) conditions, and lowest expression in lactate-sulfite (LS3) and hydrogen-sulfate (HS4) conditions. A similar expression behavior is observed for the *floxA* gene, except for the P condition where the expression is lower than in LS4. The *adh1* gene also shows highest relative expression in ES4 and P conditions.

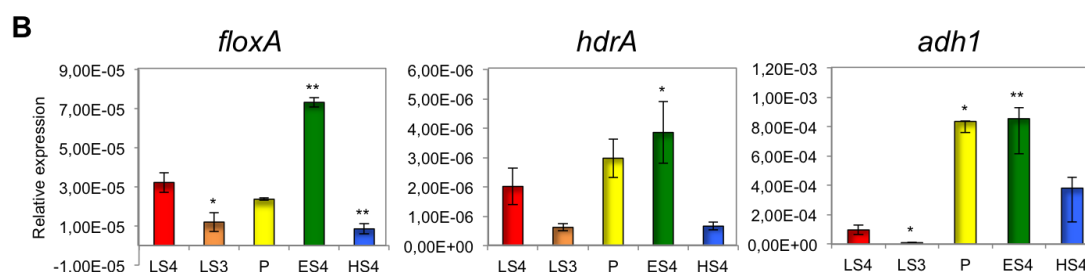


Figure 4.3 – Relative expression of *adh1*, *floxA*, and *hdrA* determined by RT-qPCR in the mid-exponential phase of cell growth either on lactate-sulfate (LS4), lactate-sulfite (LS3), pyruvate (P), ethanol (ES4), or hydrogen (HS4) containing media. The \log_{10} relative transcription levels are represented on the y-axis. The 16S rRNA gene was used as reference. The results were obtained from three independent biological replicates (means \pm standard errors). * – p -value < 0.1; ** – p -value < 0.05 (t-test).

4.1.4 – Western blot analysis of FloxA, HdrA, and Adh1 expression

To complement the RT-qPCR results, we analyzed also the expression of FloxA, HdrA, and Adh1 proteins by Western blot in *D. vulgaris* WT cells grown in the same conditions and collected at two time points: mid-exponential and beginning of the stationary phase (**Figure 4.4**). The Western blot analysis confirms that the relative protein expression levels of FloxA, HdrA, and Adh1 at middle exponential phase are highest in ES4 conditions. Again, HdrA and Adh1 also show strong expression in P conditions. Overall, there is good agreement between the mRNA levels and the protein levels for the three cases. In the stationary phase the levels of FloxA decrease, except in ES4 conditions, whereas HdrA shows a strong decrease in all conditions. In contrast, Adh1 expression is even higher at the stationary phase for ES4 and LS4 conditions.

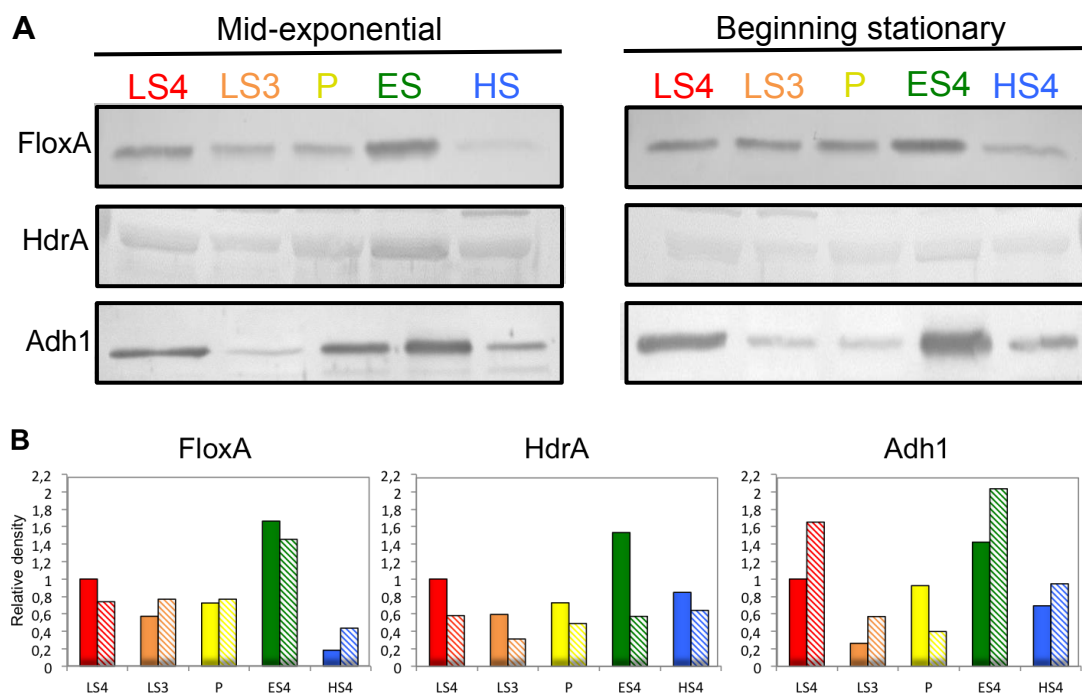


Figure 4.4 – (A) Western blot of crude cell extracts from the mid-exponential and stationary growth phase of *D. vulgaris* grown either on lactate-sulfate (LS4), lactate-sulfite (LS3), pyruvate (P), ethanol-sulfate (ES4), and hydrogen-sulfate (HS4) medium. Antibodies against Adh1, FloxA, and HdrA were used for immunodetection using 25 µg of crude extract. (B) Relative densitometry analysis comparing mid-exponential (solid pattern bar) and beginning stationary phase (diagonal pattern bar) of FloxA, HdrA, and Adh1 proteins. LS4 grown cells from the mid-exponential growth phase were used as reference.

4.2 – Studies with *dsrD*

4.2.1 – DsrD activity and interaction assays

Several attempts were made to study the direct interaction of DsrD with dissimilatory sulfite reduction enzymes (DsrAB and DsrC) and with sulfite. Firstly, enzymatic activity assays were performed in anaerobic conditions adding DsrD to DsrAB and sulfite in a reaction mixture. Then, protein-protein interaction assays were performed with BIAcore, which is based on the Surface Plasmon Resonance principle and allows the determination of affinity constants. Protein-ligand interaction assays were performed with Isothermal Titration Calorimetry, a biophysical technique that allows to determine thermodynamic parameters [binding affinity (K_d), enthalpy changes (ΔH), and binding stoichiometry (n)] of the interaction between two or more molecules in solution. Despite all these attempts no evidence was obtained to support the interaction of DsrD with DsrAB, DsrC, or even sulfite.

4.2.2 – Growth curves

In order to understand the function of the *dsrD* gene in the physiology of *D. vulgaris* cells, a *dsrD* mutant was constructed by replacing the *dsrD* gene with a kanamycin resistance cassette. Both WT and *dsrD* deletion mutant were grown in pyruvate, lactate-sulfate, and lactate-sulfite medium (**Figure 4.5**). Both strains behaved similarly during pyruvate fermentation even though the *dsrD* deletion mutant reached the stationary phase a few hours after the WT (**Figure 4.5A**). A more prominent phenotypic difference appears when both strains were cultured in lactate-sulfate medium (**Figure 4.5B**) in which the *dsrD* deletion mutant shows a great increase in the lag phase compared to the WT. After the long lag phase, the mutant strain grows to a final OD similar to the observed in the WT. Interestingly, in lactate-sulfite medium (**Figure 4.5C**), *dsrD* mutant cells were not able to grow while the WT strain reached the stationary phase after around 45 hours.

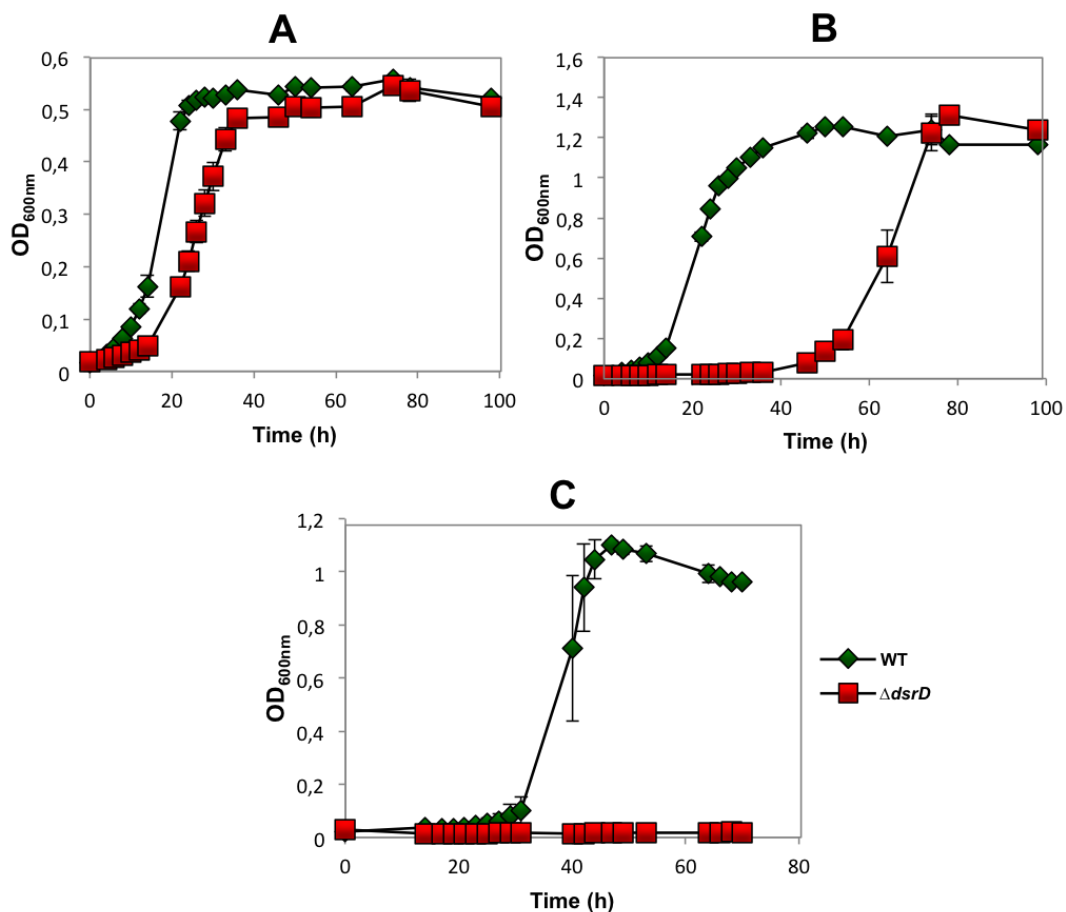


Figure 4.5 – Growth curves of *D. vulgaris* WT and *dsrD* mutant strains in (A) pyruvate fermentation, (B) lactate-sulfate, and (C) lactate-sulfite medium. The points are means of three independent growth experiments and error bars give standard deviations.

5 – Discussion

5.1 – The *hdr-flox* gene cluster

Despite the vast amount of studies concerning SRO, the energy metabolism of these organisms is still not fully elucidated. Genomic studies of SRO focusing on genes involved in energy metabolism revealed the presence of several proteins similar to enzymes of other organisms involved in FBEB, suggesting that this energy conservation mechanism is also relevant in SRO (20). FBEB is a novel mechanism of energy conservation in anaerobes, which couples an exergonic reaction to the endergonic Fd reduction (56). One example of FBEB is the HdrABC-MvhADG complex in methanogens, which couples the reduction of Fd with the reduction of CoM-S-S-CoB heterodisulfide by H₂ (**Figure 1.3**) (17). The bifurcating protein is believed to be the flavoprotein HdrA. HdrA and HdrABC proteins are found in many SRO, despite the absence of CoM-S-S-CoB heterodisulfide in these organisms (20). A new gene cluster *hdrABC-floxABCD* was found in many bacterial SRO and proposed to encode a new putative NAD(P)H dehydrogenase (FloxABCD) forming a complex with HdrABC analogous to the MvhADG-HdrABC complex of methanogens (20). The *floxA* gene codes for a FAD and NAD(P)-binding domains protein, similar to the *P. furiosus* SH subunit γ , while the *floxB* and *floxC* genes are related to *rnfC* and both code for iron-sulfur proteins similar to the *P. furiosus* SH subunit β (20). The *floxD* gene codes for a protein similar to MvhD, which in methanogens is involved in electron transfer from Mvh to Hdr (57). The *floxC* and *floxD* are fused into one single gene in several organisms, including *D. vulgaris*.

Several genomic, transcriptomic, and proteomic studies have been performed over the last years regarding the energy metabolism of SRO, using the model organism *D. vulgaris* Hildenborough and other *Desulfovibrio* species (22), (58), (59), (60), (61). The *flox-hdr* genes are often affected in these studies suggesting a relevant function in the energy metabolism, although not essential for sulfate reduction. In *D. vulgaris*, the *flox-hdr* gene cluster is present upstream of an alcohol dehydrogenase coding gene (*adh1*), which is highly expressed in *D. vulgaris* grown with lactate, pyruvate, formate, ethanol, or hydrogen as electron donors for sulfate reduction (22). A mutant lacking this gene was unable to grow in ethanol-sulfate medium indicating that Adh1 is the main enzyme catalyzing ethanol oxidation in *D. vulgaris* (22). Haveman *et al.* proposed that the *flox-hdr* genes code for a hydrogenase:heterodisulfide reductase complex involved in a hydrogen cycling mechanism (22). However, the *flox* genes do not encode for a hydrogenase subunit (20).

In this work, we studied the physiological function of the *flox-hdr* genes in *D. vulgaris*, which were demonstrated to be part of the same transcriptional unit (49). Gene and protein expression analysis of *D. vulgaris* cells grown with different electron donors for sulfate or sulfite reduction showed that the highest expression of the *floxA* and *hdrA* genes is observed when ethanol is used as electron donor (**Figure 4.3 and 4.4**). Moreover, as previously reported (62), the *adh1* gene is much more expressed than *floxA* and *hdrA*, further indicating that *adh1* is not part of the same operon as the *flox-hdr* genes. This difference in gene expression was also reflected in the protein purification: although Adh1 was purified without difficulty, we were not successful in isolating the Flox or Hdr proteins. FloxA could be easily identified by Western blot in protein purification fractions, but no corresponding band would be present in Coomassie stain of SDS-PAGE gels, which meant that the levels of FloxA present were very low. We concluded that the transcription levels of the Flox-Hdr proteins in *D. vulgaris* are low, which agrees with previous microarray results (62). Despite the generation of strain IPFG04, with a tagged FloxA we were not able to isolate the Flox-Hdr proteins in sufficient amounts for further biochemical studies, even using cells grown in ethanol.

Phenotypic studies of strains IPFG01 and IPFG02 revealed that both strains are not able to grow using ethanol as an electron donor for sulfate reduction (**Figure 4.1D**). In the complemented strain IPFG03, growth with ethanol was restored confirming that the Flox-Hdr proteins and FloxA individually, are essential in the metabolic pathway for ethanol oxidation, which agrees with their higher expression in this condition. The probable pathway involving Adh1 and Flox-Hdr proteins starts with oxidation of ethanol by Adh1 with reduction of NAD⁺ to NADH (**Figure 5.1A**). FloxA, which has a FAD and a NAD(P) binding domain, oxidizes NADH and electrons are transferred to FloxB and FloxCD. FloxCD transfers electrons to HdrABC, probably through the MvhD-like domain in FloxCD, similarly to what happens in methanogens. The electron acceptor of HdrABC remains a question mark. As suggested before (20), we propose that this electron acceptor is the cysteine containing protein DsrC (44). Since the reduction of Fd by NADH is an unfavourable reaction, and by analogy to the function of HdrABC in methanogens, we propose that the oxidation of NADH by FloxABCD involves the process of FBEB, coupling the endergonic reduction of ferredoxin by NADH to the exergonic reduction of DsrC by NADH. Regeneration of NADH involving FloxABCD-HdrABC is essential for the bioenergetics of *D. vulgaris* while growing on ethanol-sulfate, but not when the cells are growing on lactate-sulfate or pyruvate-sulfate, which agrees with the idea that pyridine nucleotides are not directly involved in the oxidation of lactate or pyruvate. We reasoned that Hdr-Flox could instead be involved in recycling NAD⁺ produced during Adh1 reduction of acetaldehyde to ethanol, formed as an alternative pyruvate fermentation product (**Figure 5.2**). Ethanol production from pyruvate as carbon and energy source has been reported before for *D. vulgaris* (63). FloxA could reduce NAD⁺ with electrons coming from Fd oxidation by HdrABC. This reaction is favorable and would not require FBEB. Fd would be reduced by the pyruvate:ferredoxin oxidoreductase (Por). Ethanol production was measured from *D. vulgaris* cells growing by pyruvate fermentation, which confirmed that the

WT strain produced significant levels of ethanol, which was not observed in the IPFG01 and IPFG02 strains, whereas ethanol production was similar in the complemented IPFG03 strain (**Figure 4.2**). These results reveal that the role of the Flox-Hdr proteins in fermentative conditions is to produce ethanol and thus recycling NAD^+ (**Figure 5.1B**). In conclusion, we showed that the recently recognized FloxABCD proteins are essential for ethanol oxidation in *D. vulgaris* and are co-expressed with the HdrABC proteins, forming a complex that we propose to perform the coupled reduction of Fd and a disulfide (the DsrC protein in SRO) using the FBEB mechanism. In pyruvate fermentation, the FloxABCD-HdrABC complex operates in reverse to reduce NAD^+ , allowing the production of ethanol and the regeneration of oxidized Fd. It is clear that the FloxABCD-HdrABC proteins are involved in the metabolism of pyridine nucleotides and thus this work contributes to a better understanding of the role of these cofactors in SRO, which has been poorly defined so far. Importantly, it provides the first link between these cofactors and sulfate (sulfite) reduction through the DsrC protein. In a broader perspective, it is also important to note that the *hdr-flox* gene cluster is not exclusive of SRO, but is widespread among many anaerobic bacteria and thus FloxABCD constitutes a novel family of NAD(P)H oxidoreductases. The presence of the *hdr-flox* gene cluster in many different Bacteria and the strictly conserved organization of the genes reveal that the so far unidentified FloxABCD proteins, together with HdrABC, perform a general and important function in the energy metabolism of these organisms. As discussed above, the function of this protein complex will be to oxidize NADH with reduction of Fd and a high-redox-potential disulfide acceptor, involving the FBEB mechanism. Thus, this work identifies a new set of proteins possibly involved in FBEB, further demonstrating the importance of this mechanism in the bioenergetics of anaerobic prokaryotes (64). Future work will focus on trying to isolate this complex from other organisms to confirm *in vitro* the FBEB process.

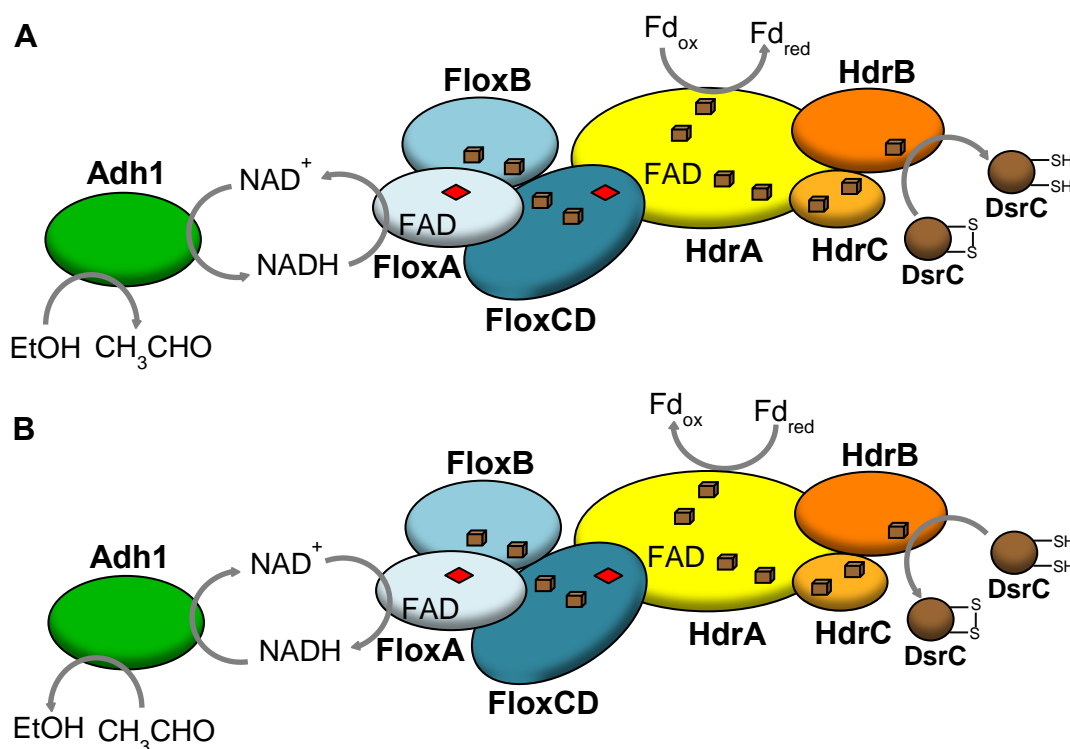


Figure 5.1 – Function of the HdrABC-FloxABCD complex. **(A)** In *D. vulgaris* growing on ethanol-sulfate, Adh1 oxidizes ethanol producing NADH that is oxidized by FloxABCD. Electrons are transferred to HdrABC, which can then bifurcate them to Fd and a second electron acceptor that we propose to be DsrC. **(B)** In *D. vulgaris* growing by pyruvate fermentation, Adh1 reduces acetaldehyde (CH_3CHO) to ethanol with NADH. The NAD^+ produced is recycled by FloxABCD, with electrons coming from Fd_{red} through HdrABC.

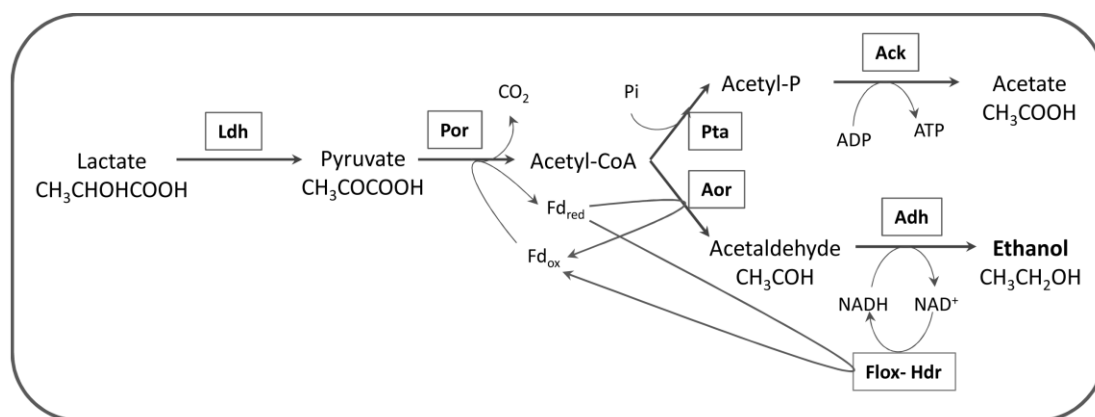


Figure 5.2 – Pathway of fermentative lactate and pyruvate oxidation in *Desulfovibrio* spp. Acetyl-CoA is mainly converted to acetyl-phosphate and this to acetate, producing ATP by substrate-level phosphorylation. In a parallel pathway, acetyl-CoA may also be reduced by aldehyde:ferredoxin oxidoreductase (63) to acetaldehyde, which is then converted to ethanol by alcohol dehydrogenase (Adh). This pathway will be more relevant in fermentative conditions. Abbreviations: Ldh – lactate dehydrogenase; Por – pyruvate:ferredoxin oxidoreductase; Pta – phosphate acetyltransferase; Ack – acetate kinase; Aor – aldehyde:ferredoxin oxidoreductase.

5.2 – Studies with *dsrD*

Despite the fact that the structure of DsrD has been obtained for some years now, the actual physiological function of this gene in SRO remains unknown (48). In *D. vulgaris* Hildenborough, *dsrD* is present in the operon *dsrABD*, in which the first two genes code for the dissimilatory sulfite reductase (DsrAB) (43). DsrAB is present in all SRO and together with DsrC is responsible for the reduction of sulfite to sulfide. Additionally, in the taurine-degrading *Bilophila wadsworthia* the *dsrD* gene is fused to the *dsrB* gene, forming a DsrB-DsrD fusion protein (45). These data suggest that DsrD may play a direct biochemical role in reduction of sulfite to sulfide. However, Hittel *et al.* have shown that DsrD binds sulfur species such as sulfite, sulfide, and sulfate with low affinity (47) and also DsrD is not found associated with DsrAB following purification. The tri-dimensional structure of DsrD reveals protein homology to DNA binding proteins and the presence of one winged-helix domain (48). Winged helix proteins, a sub-family of the helix-turn-helix protein family, contain DNA-binding domains that could bind either Z- or B-DNA. Furthermore, the proteins in the DsrD family have a well-conserved sequence (YWSSGSTT) near their C terminus which may be related to DNA binding (48).

In this work the purified recombinant DsrD was tested for interaction with DsrAB, DsrC, and sulfite both by enzymatic activity and biophysical assays (BIAcore and Isothermal Titration Calorimetry) providing no positive results. Furthermore, a deletion mutant of the *dsrD* gene was constructed. In the growth curves we observed that in comparison to the WT, the *dsrD* mutant had a long lag phase in lactate-sulfate medium, which was not detectable in pyruvate fermentation medium, therefore allowing us to conclude that *dsrD* is involved in sulfate respiration. However, after about 45 hours, the mutant strain started to grow, reaching the same OD as the WT. Korte *et al.* observed the same phenotypic phenomenon when *D. vulgaris* cells were grown in the presence of high concentrations of nitrate, an inhibitor of SRO. They concluded that nitrate resistance in WT cultures was likely conferred by spontaneous mutations, which allowed cell growth in nitrate-stress after a few days (65). This result may tell us not only that the *dsrD* deleted strain undergoes spontaneous mutations that eventually allow adaptation to sulfate medium but also that *dsrD* may be regulating *dsrAB* and hence affect the respiratory pathway. This adaptation is thought to occur in genes with redundant function as the one as *dsrD*, allowing to bypass the mutation phenotype. In sulfite growing cultures it was interesting to observe that the mutant strain was not able to grow. This may be caused by the fact that *dsrD* deletion strain is more susceptible to sulfite in high concentrations than the WT due to the importance of this gene in sulfite reduction. This interesting result tells us that *dsrD* is in fact related to sulfate/sulfite respiration, yet at which level and how that regulation is performed are still questions to be answered. Future work will focus on determining the ability of DsrD to interact with DNA *in vitro*, confirming therefore the regulatory function of this protein in the dissimilatory sulfate reduction pathway. It would also be interesting to analyze which genes substitute the function of *dsrD* allowing adaptation of the mutant strain to sulfate medium and also what is the actual contribution of these genes to the dissimilatory sulfate reduction.

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